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PB-266 223

Metabolism of Carbamate Insecticides

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Prepared for

Health Effects Research Lab, Research Triangle Park, N C

Feb 77

EPA-600/1-77-012
February 1977

PB 266 223

Environmental Health Effects Research Series

METABOLISM OF CARBAMATE INSECTICIDES

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TECHNICAL REPORT DATA
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1. REPORT NO. EPA-600/1-77-012	2.	3. RECIPIENT'S ACCESSION NO.
4. TITLE AND SUBTITLE Metabolism of Carbamate Insecticides		5. REPORT DATE February 1977
		6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) H. Wyman Dorough		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Entomology University of Kentucky Lexington, Kentucky 40506		10. PROGRAM ELEMENT NO. 1EA615
		11. CONTRACT/GRANT NO. R-802005
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, N.C. 27711		13. TYPE OF REPORT AND PERIOD COVERED
		14. SPONSORING AGENCY CODE EPA/600/11

15. SUPPLEMENTARY NOTES

16. ABSTRACT

Results of studies conducted to determine the metabolic fate of carbamate insecticides and its toxicological significance are presented. Methomyl metabolism in rats was investigated in detail as was Croneton in the rat, cow, pig and chicken. Carbaryl and carbofuran were administered to rats endotracheally either as aerosols or as components of tobacco smoke and their fate determined. Carbaryl and nitrosocarbaryl were among a series of pesticides assayed for mutagenic/carcinogenic activity using the Ames assay system. In addition, a study was conducted to determine if the in vitro metabolism of carbaryl under conditions of the Ames assay was representative of that which occurs in vivo. Respiratory ^{14}C-carbon dioxide from carbonyl-labeled carbamates was evaluated as a technique for determining the effects of various factors on the metabolism of carbamate insecticides. Carbaryl and certain of its analogs were used in studies designed to define the role of glutathione conjugation in carbamate metabolism in rats. Conjugate and boud residues of carbaryl, carbofuran, Croneton and aldicarb formed by plants were administered orally to rats and their bioavailability ascertained. Biliary excretion and enterohepatic circulation were considered in addition to urinary and fecal elimination.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS		b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Insecticides Carbamates Toxicity Metabolism In Vivo In Vitro	Pesticides Chemical analysis	Carbamate Inseciticides Conjugates	07 C 06 F, T

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS *(This Report)* UNCLASSIFIED	21. NO. OF PAGES 257
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE PC A12 MF A01

EPA Form 2220-1 (9-73)

EPA-600/1-77-012
February 1977

METABOLISM OF CARBAMATE INSECTICIDES

by

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Grant No. R-802005

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This project was conducted to meet program needs on the fate of carbamate insecticides. These results provide information on the rates of metabolite degradation, sites and levels of storage of the metabolites and their degradation products in animals.

JHKnelson

John H. Knelson, M.D.,
Director
Health Effects Research Laboratory

ABSTRACT

Studies were conducted to determine the metabolic fate of carbamate insecticides and to evaluate factors which influence metabolite formation and their toxicological significance. Methomyl metabolism in rats was investigated in detail while Croneton was studied in the rat, cow, pig and chicken. Several insecticides, including carbaryl and carbofuran, were administered to rats endotracheally either as aerosols or as components of tobacco smoke and their fate determined. In the smoke experiments, the quantity and nature of the residues inhaled were established by analysis of mainstream smoke from cigarettes fortified with radioactive insecticides. Carbaryl and nitrosocarbaryl were among a series of pesticides assayed for mutagenic/carcinogenic activity using the Ames assay system. In addition, a study was conducted to determine if the *in vitro* metabolism of carbaryl under conditions of the Ames assay was representative of that which occurs *in vivo*. The production of respiratory ^{14}C-carbon dioxide from carbonyl-labeled carbamates was shown to be an excellent quantitative method for evaluating the effects of various exogenous and endogenous factors on the metabolism of carbamate insecticides. Carbaryl and certain of its analogs were used in studies designed to define the role of glutathione conjugation in carbamate metabolism in rats. Conjugate and bound residues of carbaryl, carbofuran, Croneton and aldicarb formed by plants were administered orally to rats and their bioavailability ascertained. Biliary excretion and enterohepatic circulation were considered in addition to urinary and fecal elimination.

This report was submitted in fulfillment of Grant Number R-802005 by the University of Kentucky under the sponsorship of the Environmental Protection Agency. Work was completed as of October 1976.

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ACKNOWLEDGMENTS

The principal investigator wishes to acknowledge the efforts and dedication of those students, post-doctoral fellows, and technical assistants who have contributed so much to this research program on carbamate insecticides. Sincere thanks are extended to those individuals listed below for their hard work and cooperation during the course of these investigations.

Graduate Research Assistants

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I CONCLUSIONS

Hydrolysis, oxidation and conjugation are biochemical reactions common to all insecticidal carbamates. Ester hydrolysis almost invariably reduces toxicity, while oxidation may result in compounds retaining a high degree of toxicity. Cronaton, for example, undergoes sulfur oxidation to the sulfoxide which is more acutely toxic to rats and mice. The sulfone derivative is of similar toxicity as the parent compound. Methomyl metabolism in rats involves isomerization and Beckmann rearrangement in addition to the other reactions common to the carbamates. Carbamate insecticides entering the body via inhalation are metabolized similarly to when ingested. When present as residues in tobacco, much of the intact carbamate is transferred to the smoker by volatilization before being degraded during the actual burning process.

Nitrosocarbaryl is a potent direct mutagen when assayed in the Ames bacterial system. Carbaryl is inactive either with or without the rat liver enzymes. In the presence of the liver enzymes, carbaryl is almost completely metabolized within one hour to products commonly formed in the *in vivo* mammalian system. Thus, the metabolites as well as the parent compound were assayed for their mutagenic potential.

Water soluble metabolites of carbamates formed in bean plants are rapidly absorbed from the gut of rats and are excreted almost quantitatively in the urine even though biliary excretion is extensive. The bound residues in the plant are not bioavailable as evidenced by complete fecal excretion without detection of residues in the bile. Reports that glutathione conjugation plays a major role in carbaryl metabolism in rats were not substantiated. It appears that the proposed mercapturic acid derivatives of carbaryl were artifacts resulting from strong acid treatment of the dihydrodiol metabolites of carbaryl.

II RECOMMENDATIONS

Each carbamate insecticide must be subjected to thorough metabolic studies with an awareness on the part of the investigator that metabolic pathways are not always predictable. Moreover, reviewers of metabolism studies, whether for publication or regulatory purposes, should look specifically for evidence suggesting the existence of metabolites not reported, and/or of intermediates which obviously must exist in order to yield the products identified. Such scrutiny of the data would lessen considerably the likelihood of a compound, as was the case with methomyl, being evaluated on the basis of proposed metabolic pathways inconsistent with the experimental evidence.

Efforts should be made to more fully define the toxicological significance of the conjugated carbamate metabolites. Unlike the bound residues, the conjugates are bioavailable in animals and may be converted *in vivo* to toxic carbamate materials. Chronic toxicological evaluations should be conducted on individual conjugate metabolites if they constitute a significant portion of the terminal residues in products destined for human consumption.

Carbamate metabolites in animal and plant tissues should be classified as bound residues only if demonstrated to be nonbioavailable when administered orally to appropriate experimental animals. Such residues occurring in substrates such as feed of dairy animals and crops returned to the soil should be investigated to determine if the bound residues are converted by microbial action, etc., to a bioavailable form and persist as such in products consumed by man.

While there are many problems yet to be resolved relative to carbamate metabolism, this group of chemicals remains a viable source of new pest control agents. Their many desirable features justify continued efforts to expand the utility of this very important family of insecticide.

III EXPERIMENTAL

INTRODUCTION

Carbamate insecticides as we know them today had their start in the late 1950's with the introduction of carbaryl (1-naphthyl N-methylcarbamate, Trade Name Sevin). The compound was rapidly accepted on the world market because of its efficacy, moderate mammalian toxicity, and relatively low persistence in the environment. From the metabolic point, carbaryl was assumed to undergo simple ester hydrolysis to yield innocuous products.

Studies in the 1960's and early 1970's demonstrated that carbaryl metabolism was far more complex than originally reported and, in fact, was among the most complex ever encountered with a pesticidal compound. Even today, carbaryl metabolism is the topic of many investigations, either as a model compound or in attempts to fully define its metabolic fate. In the interim, other carbamates having vastly different chemical and toxicological properties than carbaryl have been introduced. One commonality persisted, however, and that was the complexity of metabolism involving predominately hydrolytic, oxidative and conjugative reactions (Kuhr and Dorough 1976).

Over the past 15 years a major portion of the principal investigator's research program has been devoted to carbamate metabolism and its significance relative to the safe use of these insecticides. Among those compounds studied most extensively are carbaryl, aldicarb, carbofuran and methomyl, each of which is currently widely used for insect control. Since 1970, much of the work on carbamate metabolism has been funded from a grant from the Environmental Protection Agency (Grant Number R-802005). A progress report (identified as EPA-650/1-74-002, September 1973) was submitted covering the period of September 1, 1970 through August 31, 1973. A renewal of the grant was requested and approved for the period of February 1, 1974 through January 31, 1977. Progress made during the latter project period is contained in the present report.

The principal objective of the proposed research was to define the metabolic fate of carbamate insecticides in various biological systems so that this information could be used by regulatory officials in assessing the safety of the compounds involved. During the process, studies were performed which would contribute to ascertaining the significance of metabolites encountered with the carbamates. Studies wherein physiological and biochemical processes responsible for variations in carbamate metabolism were also explored.

Those studies performed during the past three years towards attaining the objectives of the project are presented below. Studies essentially completed in 1973 and the results included in Report Number EPA-650/1-74-002 have not been repeated herein. However, those conducted since 1973, even if published elsewhere, are made a part of the present report to provide a single source of reference for those investigations supported by EPA Grant Number R-802005.

Research supported by this EPA grant has contributed enormously to papers by the principal investigator and his colleagues which are not experimental in nature. A book on carbamate insecticides published in 1976 (Kuhr and Dorough) resulted from an invitation by CRC Press to the principal investigator based on his experience with the carbamates, much of which was obtained while funded by EPA. A similar situation resulted in a presentation to the Pesticide Division of ACS on the biological activity of pesticide conjugates (Dorough 1976), and to the 1976 Gordon Conference on Drug Metabolism on the topic of bioavailability of covalently bound pesticide residues. Another such occurrence was the preparation of a review article dealing with the state-of-the-art of carbamate residue analysis (Dorough and Thorstenson 1975). While the aforementioned papers are not in the present report, they deal largely with carbamate insecticides and are based on the data which are reported.

METABOLISM OF METHOMYL IN RATS

Since the preliminary report of methomyl {S-methyl-N-[(methylcarbamoyl) oxy]thioacetimidate} metabolism by Baron in 1971, and the detailed reports in 1973 (Harvey and Pease, 1973; Harvey and Reiser, 1973; Harvey et al., 1973), we have been intrigued by the metabolic pathway proposed for this oxime carbamate in animals, plants, and soils. Using methomyl radiolabeled in the $^{14}C=N$ position, the radioactive metabolic products observed were simply carbon dioxide and acetonitrile. Wile some difficulties were apparent in the quantitative collection of the volatile metabolites, the qualitative evidence was sound in each of the aforementioned studies.

A particularly interesting point concerning these results was the chemical route leading to the radioactive carbon dioxide and acetonitrile. This point was not raised by any of the authors and may have been considered self-evident. Nonetheless, their mechanism of bioformation was not readily obvious to us, possibly because of our experience with aldicarb metabolism (Dorough, 1970).

With aldicarb, the compound is metabolized similarly by most organisms. Hydrolysis of the carbamate ester is usually very rapid, especially in animals, and has been documented by the release of [^{14}C] carbon dioxide from [^{14}C] carbonyl-labeled aldicarb. The studies with methomyl did not include a carbonyl radiocarbon so it could not be determined if the carbon dioxide and acetonitrile formed from [$^{14}C=N$] methomyl came from intermediates containing the intact ester linkage or were formed subsequent to hydrolysis.

Sulfur oxidation to form the sulfoxide (SO) and sulfone (SO_2) of aldicarb and/or its oxime constituted the major metabolic reactions in all systems tested. With methomyl, there was no indication that sulfur oxidation was involved at all in its metabolism. Thus, the real difference in metabolism appears to be in the fate of the oxime hydrolysis products of aldicarb and methomyl. This, of course, requires the assumption that methomyl is hydrolyzed rapidly as is aldicarb and other N-methylcarbamate insecticides.

Considering the chemical differences which might account for the different metabolic pathways of aldicarb and methomyl, the most obvious was the manner in which the thioalkyl groups are bonded to the imide carbon. With aldicarb, the thioalkyl moiety is attached to the carbon alpha to the imide carbon and is designated chemically as an alkylthioether. The thioalkyl group of methomyl is bonded directly to the imide carbon and is classified as a thiohydroximate ester. That these differences could account for the metabolic differences for aldicarb and methomyl was supported by reports on the metabolism of DS-15647 (Whitten and Bull, 1974) and thiocarboxime (Hutson, 1971). DS-15647, an alkylthioether, was metabolized by hydrolysis and/or sulfur oxidation in the same manner as aldicarb, whereas thiocarboxime, a thiohydroximate ester, was metabolized to carbon dioxide and acetonitrile as was methomyl. Thus, it might be speculated that the metabolic pathways of alkylthioether and thiohydroximate oxime carbamates follow different metabolic schemes. This being the case, the mechanism of methomyl metabolite formation, if defined, would likely apply also to other insecticidal thiohydroximate esters.

Materials and Methods

Chemicals - Radioactive materials used in this study are shown in Table 1 along with the position of the radiolabel and the abbreviated designations used throughout this paper. [^{14}C=O] Methomyl was prepared from syn methomyl oxime and methyl [^{14}C] isocyanate and purified by tlc. Its specific activity was 5.8 mCi/mmol. syn [^{14}C=N] Methomyl oxime and syn [^{14}C=N] methomyl were synthesized from [^{14}C] acetonitrile using the method of Harvey et al. to give a specific activity of 1.4 mCi/mmol. The syn [^{14}C=N] oxime was converted to the anti [^{14}C=N] isomer by ultraviolet irradiation and the anti [^{14}C=N] methomyl formed

Table 1. RADIOLABELED MATERIALS USED IN THE STUDY OF METHOMYL METABOLISM IN RATS

Chemical Name	Abbreviations
syn S-Methyl N[(methylcarbamoyl-carbonyl-^{14}C)oxy]thioacetimidate	*syn*[^{14}C=O]methomyl
syn S-Methyl [1-^{14}C]N-[(methyl-carbamoyl)oxy]thioacetimidate	*syn*[^{14}C=N]methomyl
anti S-Methyl[1-^{14}C]N-[(methyl-carbamoyl)oxy]thioacetimidate	*anti*[^{14}C=N]methomyl
syn S-Methyl[1-^{14}C]N-hydroxythio-acetimidate	*syn*[^{14}C=N]methomyl oxime
anti S-Methyl[1-^{14}C]N-hydroxythio-acetimidate	*anti*[^{14}C=N]methomyl oxime
Ethyl di-n-propylthiocarbamate-^{14}C carbonyl	[^{14}C=O]carbonyl Eptam

by reacting it with methyl isocyanate.

Confirmation of structures was accomplished by comparing infrared and 'H nmr spectra of the compounds with those previously reported (Davies et al., 1968). Radioactive purity of the syn preparations, as determined by tlc and autoradiography, was 95% or greater. anti [^{14}C] Methomyl contained some of the syn isomer, up to 20%, when administered to test animals because of the ease with which it was converted to the syn form. anti Oxime could be resolved by tlc from the other radioactive components in the crude preparation mixture, but rechromatography after extraction from the gel showed varying amounts of syn oxime and three to four unknown products. This instability prevented direct structural confirmation by spectral analysis and its conversion to the anti methomyl when reacted with methyl isocyanate was accepted as proof of structure.

[^{14}C] Carbonyl-labeled Eptam (ethyl di-n-propylthio [^{14}C] carbamate), specific activity 6 mCi/mmol, was prepared from radioactive phosgene following the method of Tilles (1959). Nonradioactive methomyl, methomyl oxime and Eptam used as standards were analytical grade materials from our residue laboratory.

Thin Layer Chromatography - Silica gel chromatoplates (Analtech, 250µ) were used to resolve the syn and anti isomers of methomyl and its oxime derivatives. The carbamate forms were separated by developing the chromatoplates in ethyl acetate (syn-methomyl R_f 0.56, anti-methomyl R_f 0.67). Effective separation of the two oxime forms was not accomplished in this solvent system but the compounds were resolved by developing the plates in a 5 to 1 dichloromethane and ether mixture (syn-oxime R_f 0.71, anti-oxime R_f 0.88). Methomyl (R_f 0.16) was separated from the oxime (R_f 0.67) using a 9 to 1 mixture of ether and hexane as the solvent system. Isomeric configuration of the respective compounds was then determined by tlc analysis in the appropriate solvent system just described. Radioactive materials on the chromatoplates were detected by autoradiography using Kodak no-screen medical X-ray film. Nonradioactive standards were viewed under short wave ultraviolet light.

Treatment - Albino rats (Cox-SD, Laboratory Supply Co.) weighing approximately 250 g each were treated with from one to two million dpm's of the radioactive compound used in each experiment. For oral dosing, the chemical was dissolved in 0.5 ml of corn oil and administered to the rats via a feeding needle. All treatments were by the oral route except in one experiment where syn- and anti-oxime were injected intraperitoneally into the animals. Here, only one animal was used for each compound, whereas all oral treatments consisted of a minimum of two rats from which each set of data was obtained. Numerous animals were treated for the sole purpose of developing and evaluating methods of collecting radioactivity in the expired gases. Quantitative data in this report do not contain the results obtained in the methodology experiments, but it is noteworthy that they invariably substantiated the results which are presented.

Radioassay - Quantitation of radioactivity was accomplished by liquid scintillation counting (Packard Tri-Carb 3385/544) using a premixed scintillation fluid (3a70B, Research Products International, Elk Grove Village, IL). Aliquots from traps used for the collection of respiratory [^{14}C] gases, and samples of rat urine were radioassayed directly. Feces samples, approximately 0.5 g, were combusted using a Packard Model 306 oxidizer, and the liberated [^{14}C] carbon dioxide trapped in a 2 to 1 mixture of 2-methoxyethanol and 2-aminoethanol for radioassay.

Collection of Samples - Treated animals were placed in glass metabolism cages which allowed separate collection of the urine and feces. To collect radioactive materials in the respiratory gases, air (about 100 ml/min) was passed through the cage into one or more solutions designed for the trapping of carbon dioxide and/or acetonitrile. When monitoring only for carbon dioxide and for relatively short periods, 6-12 hrs, the trap consisted of 20 ml of a 2 to 1 mixture of 2-methoxyethanol and 2-aminoethanol (System A).

Separate collecting of respiratory carbon dioxide and acetonitrile was accomplished by passing the air from the animal cage through two cold acetone (dry ice-acetone) traps to collect the acetonitrile and then through 100 ml of 4 N sodium hydroxide for the collection of carbon dioxide (System B). While this system effectively trapped the compounds in question, there remained the possibility that other radioactive materials could be present in the respiratory gases of [^{14}C=N] methomyl-treated animals. This possibility was evaluated by using a third trapping system (System C) whereby the air was directed from the cage into 4 N sodium hydroxide, and from there it was mixed with oxygen, 200 ml/min, and passed through a Beckman Biological Materials Oxidizer (BMO). The purpose of the BMO was to combust any radioactive components escaping the hydroxide trap, including acetonitrile, to carbon dioxide which was then collected in a second hydroxide trap. To account for the [^{14}C] acetonitrile collected in the trap placed before the BMO, barium hydroxide was added to the 4 N sodium hydroxide to precipitate the radioactive carbonate. The trap solution was centrifuged, the pellet radioassayed as an indication of [^{14}C] carbon dioxide in the trap, and the supernatant radioassayed to determine the [^{14}C] acetonitrile content of the trap solution.

Acetonitrile Confirmation - Radioactivity collected in the cold acetone traps from the respiratory gases of rats treated with [^{14}C=N] methomyl was assumed to be $CH_3\,^{14}C\equiv N$ (Harvey et al., 1973). Confirmation of its identity was attempted by determining if the radioactivity could be converted to acetophenone [$C_6H_5-^{14}C(O)CH_3$]. The latter has been prepared from authentic acetonitrile (Shriner and Turner, 1930) and, therefore, the procedure was used for the attempted synthesis of the radioactive form from the methomyl metabolite suspected of being $CH_3\,^{14}C\equiv N$. The starting material consisted of 3 x 10^5 dpm of the metabolite in about 50 ml of acetone and 10 ml of nonradioactive acetonitrile.

Following distillation to a remaining volume of 6 ml for the complete removal of acetone, the semicarbazone derivative of actophenone [C_6H_5-$C(CH_3)NNHC(O)NH_2$] was prepared and recrystallized from ethanol to a constant melting point and until a constant specific activity was, or was not, evident.

Results

Trapping of [^{14}C] Volatiles - Trapping of [^{14}C] carbon dioxide in the respiratory gases of animals treated with [$^{14}C=O$] methomyl was essentially quantitative using a mixture of 2-methoxyethanol and 2-aminoethanol (Table 2, System A). It did not effectively trap [^{14}C] acetonitrile and thus could be used to selectively monitor carbon dioxide if so desired. In studies using *syn* [$^{14}C=N$] methomyl, which yields both radiolabeled compounds, the amount of [^{14}C] carbon dioxide collected in System A was the same as that in systems allowing separate quantitation of carbon dioxide and acetonitrile.

With trapping System B, the acetonitrile collected in the cold acetone, primarily in trap I, while only small quantities of carbon dioxide, 3% or less of that generated, were in trap III, a 4 N sodium hydroxide solution.

The incorporation of the BMO combustion apparatus into the trapping system (System C, Table 2) was an attempt to quantitate total radioactivity in the respiratory gases. Without trap I, the carbon dioxide as well as other components converted to carbon dioxide in the BMO would collect in trap II, giving a single value for respiratory [^{14}C] components. Of course, the carbon dioxide was removed by the sodium hydroxide trap I, but the solution also contained almost one-half of the acetonitrile passing through the system. The most important point relative to System C was that radioactive acetonitrile entering the BMO was combusted and collected as carbon dioxide without any appreciable loss. This suggested that other radioactive components of the

Table 2. EFFICIENCIES OF VARIOUS TRAPPING SYSTEMS FOR COLLECTION OF RADIOACTIVE CARBON DIOXIDE AND ACETONITRILE IN THE RESPIRATORY GASES OF RATS TREATED WITH [^{14}C] METHOMYL.

		% of total ^{14}C passed through traps[b]	
	Trapping system[a]	Carbon dioxide	Acetonitrile
A.	Trap I, 2:1 2-methoxyethanol-2-aminoethanol	100	1
B.	Trap I, Acetone, -10 to 0°C	2	92
	Trap II, Acetone, -10 to 0°C	1	8
	Trap III, 4 N Sodium Hydroxide	97	0
C.	Trap I, 4 N Sodium Hydroxide[c]	100	45
	Combustion apparatus, then		
	Trap II, 4 N Sodium Hydroxide	0	55

[a] Air from animal was passed through traps in order listed under each system.

[b] For recovery experiment, $^{14}CO_2$ was generated by treating animals with [^{14}C=O] methomyl; $CH_3{}^{14}C{=}N$ was volatilized from a water solution by bubbling air through the water and then into the indicated traps.

[c] Air from first NaOH trap was mixed with oxygen, directed through a Beckman Biological Materials Oxidizer, and then into the second NaOH trap.

expired air, if any, would be similarly quantitatible. The fact is, however, that respiratory [^{14}C] gases resulting from treatment of rats with [^{14}C=N] methomyl were of the same magnitude whether trapped in System B or System C. These results demonstrated that there was no radioactivity in the expired air which escaped System B and was trapped in System C, and provided strong evidence that System B effectively trapped the total respiratory radiocarbon liberated by the animals.

Nature of [^{14}C] Volatiles - That radioactivity which could be trapped by 2-methoxyethanol:2 aminoethanol and by 4 N sodium hydroxide, and which could be precipitated from the latter with barium hydroxide was characterized as carbon dioxide. The remainder of the radiocarbon in the expired air of rats was identified as acetonitrile based upon its synthesis to acetophenone semicarbazone. The final product, when recrystallized to a constant melting point and specific activity, contained 83% of the radioactivity added to the initial reaction mixture from traps I and II of trapping system B (Table 2).

Fate of syn [^{14}C=O] Methomyl - Orally administered syn [^{14}C=O] methomyl to rats was rapidly hydrolyzed as indicated by the evolution of [^{14}C] carbon dioxide in the expired air (Table 3). While the rate of ester hydrolysis appeared somewhat faster in females than in males during the early stages of the experiment, there was little difference in the total hydrolysis after 24 hr (87.7% of dose in females and 83% in males). After the same period following treatment, the urinary radiocarbon accounted for approximately 5% of the administered dose while only 0.2% was voided in the feces. The average total recovery considering all treated animals was 90.4% of the dose.

Tlc analysis of concentrated urine applied directly to the chromatoplates and developed in ethyl acetate showed that none of the radioactivity moved from the origin. Thus, the radiocarbon was not in the form of the parent carbamate or its sulfoxide and sulfone analogs. These results agreed with those earlier reported for methomyl and for

Table 3. FATE OF RADIOCARBON FROM RATS TREATED ORALLY WITH SYN [^{14}C=O] METHOMYL

Nature of ^{14}C and hrs after dose	Cumulative % of dose[a]	
	Female	Male
Carbon dioxide		
1	6.0	13.5
2	33.9	27.0
4	69.5	55.0
6	84.0	79.0
10	87.4	81.0
24	87.7	83.0
Urinary-^{14}C, 24	4.9	4.8
Fecal-^{14}C, 24	0.2	0.2
Total Recovery, 24	92.8	88.0

[a] Each value is the average from two test animals.

thiocarboxime. Because of the very low level of urinary [^{14}C] metabolites, no attempt was made to further characterize the material.

Fate of [^{14}C=N] Methomyl and Oxime - Having established in the methodology experiments that [^{14}C] acetonitrile was expired in the air of rats treated with syn [^{14}C=N] methomyl, it was considered essential to determine if orally administered [^{14}C] acetonitrile would similarly be expired. Data in Table 4 show that this indeed was the case and that it was detectable in the expired air after only 1 hr. By 6 hr, almost one-half of the administered dose had been expelled by this route. A small amount of the dose, 9%, was trapped as [^{14}C] carbon dioxide but its formation was restricted to the first 2 hr following treatment. The importance of these data, as will become more evident in the results presented below, was the documentation that [^{14}C] acetonitrile was rapidly and efficiently voided from the rats in the respiratory gases; therefore, a delay in the detection of acetonitrile from syn [^{14}C=N] methomyl-treated rats should be indicative of its rate of formation and not of its rate of elimination.

As just suggested, there was a considerable delay in the formation of acetonitrile in rats treated with syn [^{14}C] methomyl (Table 5). The compound first occurred in the trap solution between 4 and 6 hr after treatment, but then its rate of formation increased so that after 24 hr, respiratory [^{14}C] acetonitrile accounted for 11% of the dose. [^{14}C] Carbon dioxide, on the other hand, was detected in the first assay of the trap, 2 hr after treatment, in quantities equivalent to 2.5% of the dose. Its continued liberation over the 24 hr test period resulted in 19.5% of the dose being expired as carbon dioxide. Collection of respiratory radiocarbon from one of the animals for 72 hr showed that the carbon dioxide increased to 24.4% of the dose, while acetonitrile increased to 14.9%. The pattern of elimination of these products from syn [^{14}C=N] methomyl was the same as that reported by Harvey et al. (1973).

Table 4. RADIOCARBON IN RESPIRATORY GASES OF RATS TREATED ORALLY WITH $^{14}C\equiv N$ LABELED ACETONITRILE

	Cumulative % of dose	
Hours after treatment	Carbon dioxide	Acetonitrile
1	7.1	0.6
2	9.1	10.3
4	9.1	27.7
6	9.1	48.7

Table 5. [^{14}C] CARBON DIOXIDE AND [^{14}C] ACETONITRILE RELEASED IN THE RESPIRATORY GASES OF FEMALE RATS TREATED WITH [^{14}C=N]-LABELED METHOMYL OR METHOMYL OXIME

Hours after treatment	Cumulative % of dose					
	anti-[^{14}C=N] Methomyl		*syn*-[^{14}C=N] Methomyl		*syn*-[^{14}C=N] Oxime	
	CO_2	CH_3CN	CO_2	CH_3CN	CO_2	CH_3CN
2	1.3	3.0	2.5	0	2.5	0
4	3.9	5.8	6.0	0	7.0	0.3
6	5.0	8.4	8.5	0.7	10.5	0.4
10	6.3	14.0	12.0	3.0	14.0	0.8
14	7.1	18.4	15.5	5.5	17.0	1.0
22	7.4	25.1	19.0	10.5	21.0	2.2
24	7.6	27.9	19.5	11.3	21.8	2.4

Radiocarbon in the expired air of rats treated with anti [^{14}C=N] methomyl was predominately acetonitrile, 27.9% of the dose after 24 hr, and it was readily apparent after only 2 hr (Table 5). [^{14}C] Carbon dioxide was eliminated in only small amounts, 7.6% of the dose after 24 hr, with the majority generated in the first 6 hr. Because the anti methomyl contained up to 20% of the syn isomer, it is likely that most of the $^{14}CO_2$ was derived directly from this source. In any event, the data do show that anti methomyl was metabolized differently than syn methomyl, and pointed up the possibility that [^{14}C] acetonitrile from the syn methomyl treatment was generated from the anti form following *in vivo* isomerization.

syn [^{14}C=N] Oxime, unlike its carbamate derivative, was not metabolized appreciably to acetonitrile (Table 5). However, the amount of $^{14}CO_2$ formed from the oxime was about the same as for syn methomyl. This same situation was observed in studies of the metabolism of thiocarboxime and its oxime in rats (Hutson et al., 1971). Apparently, the syn oxime is more resistant to reactions resulting in acetonitrile than is the syn carbamate and, thus, follows the metabolic route which yields carbon dioxide. Because the anti [^{14}C=N] oxime was even more unstable than anti methomyl, it was not included in the oral dose studies.

The anti [^{14}C=N] oxime preparation was included in a cursory examination of the fate of the oximes when injected intraperitoneally into rats. Results of these experiments showed that the anti [^{14}C=N] oxime, while impure, formed 20 times more [^{14}C] acetonitrile than did the syn isomer (Table 6). Carbon dioxide formation from the injected syn [^{14}C=N] oxime was less than that resulting from oral administration of the compound (Table 5). However, the quantities of [^{14}C] acetonitrile were similar for both routes of treatments. Even with the difference in carbon dioxide expired, the data were sufficiently similar to show that the degradation observed was not a function of reactions unique to the gut. The relatively large amount of [^{14}C] carbon dioxide formed from anti [^{14}C=N] oxime, 17.4% of the dose after 24 hrs, was incon-

Table 6. [^{14}C] ACETONITRILE AND CARBON DIOXIDE IN THE RESPIRATORY GASES OF RATS TREATED BY IP INJECTION WITH [^{14}C=N]-LABELED ANTI AND SYN METHOMYL OXIME

	Cumulative % of dose			
	anti-[^{14}C=N] Oxime		syn-[^{14}C=N] Oxime	
Hours after treatment	CO_2	CH_3CN	CO_2	CH_3CN
6	10.2	4.9	6.2	0.3
14	13.8	12.1	11.6	0.7
24	17.4	20.5	16.5	1.7

sistent with the amount, 7.6%, resulting from treatment of animals with *anti* [^{14}C=N] methomyl (Table 5). Presumably, this resulted from the several impurities, including *syn* oxime, contained in the *anti* methomyl preparation.

Urine of rats treated orally with [^{14}C=N] methomyl and with *syn* [^{14}C=N] oxime contained from 25 to 35% of the administered radioactivity after 24 hr. The chemical nature of the urinary [^{14}C] metabolites was not critically evaluated. However, it was obvious that not more than 5% of the methomyl dose was excreted in a form containing the carbonyl carbon (Table 3) and, therefore, this represented the maximum amount possible in the carbamate form. Concentration by freeze-drying of urine from rats treated with *syn* [^{14}C=N] methomyl resulted in 20% loss of the radiocarbon, indicating that a portion of the [^{14}C] components, possibly acetonitrile, was very volatile. That radioactivity in the urine concentrate was not moved from the tlc origin when developed in ethyl acetate. With thiocarboxime, a compound of similar structure to methomyl, the urinary metabolites were primarily glucoside and sulfate conjugates of the oxime.

Proposed Route of $^{14}CO_2$ and $CH_3\ ^{14}C \equiv N$ Production from syn [^{14}C=N] Methomyl - Prior to the experiments with *anti* [^{14}C=N] methomyl, the possibility that [^{14}C] acetonitrile and [^{14}C] carbon dioxide were derived from an intermediate metabolite of *syn* [^{14}C=4] methomyl capable of undergoing two separate routes of degradation was considered. This was based on reports that both methomyl and methomyl oxime are more stable in the *syn* form and the absence of any evidence for the biological isomerization of the compounds, or of the metabolic products of the *anti* isomers if formed. The metabolic data obtained with the *anti* form of this carbamate showed, however, that such a metabolite probably did not exist. Production of both acetonitrile and carbon dioxide could be explained if *syn* methomyl or its oxime was partially converted, *in vivo*, to the *anti* isomer. That is, [^{14}C] acetonitrile could be formed from the *anti* isomer and [^{14}C] carbon dioxide from the *syn* isomer.

Because of the rapid hydrolysis rate of methomyl (Table 3), it was evident that acetonitrile and carbon dioxide were not derived directly from intermediates containing the intact carbamate ester. The majority of these volatile metabolites were formed after 80% or more hydrolysis of the carbamate had occurred. Studies with the *syn* and *anti* oximes (Table 5 and 6) demonstrated that these metabolites of methomyl hydrolysis could serve as primary intermediates to carbon dioxide and acetonitrile, respectively. The study with *syn* oxime also showed that this isomer was relatively stable to isomerization in the rat; only 2.4% of the dose was expired as acetonitrile. Thus, the major source of *anti* oxime in the *syn* methomyl-treated rats was apparently from hydrolysis of *anti* methomyl formed by isomerization of the administered carbamate.

The conversion of *syn* methomyl to the *anti* form obviously was not an instantaneous reaction in the rat. If that had occurred, the presence of acetonitrile would have been evident in the expired air immediately after treatment and this was not the case (Table 5). This is based on the findings that acetonitrile, per se, was expired very rapidly (Table 4) and that *anti* methomyl yielded acetonitrile at a rate almost identical to the rate of carbon dioxide formation from *syn* methomyl. Therefore, the delay in acetonitrile formation from *syn* methomyl treatments probably resulted from the time required for its precursors, *anti* methomyl and *anti* oxime, to be generated in sufficient quantities to allow biosynthesis of acetonitrile in detectable quantities.

While the *anti* oxime preparation gave considerable quantities of carbon dioxide (Table 6), indicating isomerization to the *syn* form, its formation was probably from impurities in the preparation and not from *in vivo* formation of *syn* oxime. A better estimate of the *in vivo* stability of the *anti* oxime may be gained through analysis of results obtained in the *anti* [^{14}C=N] methomyl rat study (Table 5). While hydrolysis to the *anti* oxime and subsequent metabolism to acetonitrile were evident after just 2 hr, and continued progressively for 24 hr, where it equalled 27.9% of the dose, there was very little [^{14}C] carbon dioxide given off by the animals. Possibly, the carbon dioxide was formed largely from *syn* methomyl, up to 20%, present in the *anti* methomyl rather than resulting entirely from the isomerization of *anti* methomyl or its oxime. Support for this was indicated by treating a female rat with *anti* [^{14}C=N] methomyl containing only 5% of the *syn* form. After 24 hr, 34% of the dose was expired as [^{14}C] acetonitrile and only 5% as [^{14}C] carbon dioxide. The conclusion is, therefore, that little *in vivo* isomerization takes place with *anti* methomyl or with *anti* oxime.

Having concluded that *syn* [^{14}C=N] methomyl was isomerized, in part, to the *anti* isomer in the rat, and that the carbamates were then hydrolyzed to their respective oximes which, in turn, were degraded to carbon dioxide and acetonitrile, possible mechanisms for the latter reactions were considered. The nature of the reactants and end products suggested that Beckmann type rearrangements were involved in the metabolic degradation of the oximes. Such chemical reactions have been reported for *syn* and *anti* methomyl oxime (Davies et al., 1968) and provide the basis for the proposed *in vivo* mechanism for the formation of carbon dioxide and acetonitrile. Under acid conditions, the *anti* isomer underwent rearrangement to yield acetonitrile, a product of its metabolism in the rat. The *syn* isomer underwent rearrangement to form S-methyl N-methylthiocarbamate. Quite clearly, the S-methyl N-methylthiocarbamate formed from *syn* oxime by Beckmann rearrangement was not the respiratory product of *syn* oxime metabolism in the rat. It was, however, a possible precursor to that end product, carbon dioxide. Evidence that a [^{14}C] carbonyl-labeled thiocarbamate is metabolized to [^{14}C] carbon dioxide in rats was obtained with Eptam. This herbicide was metabolized very rapidly to [^{14}C] carbon dioxide and after 8 hr accounted for 35% of the administered radiocarbon. The metabolic conversion of the carbonyl carbon of thiocarbamates to carbon dioxide was previously proposed (Ong and Fang, 1970), but had not been demonstrated experimentally. With this evidence, the final step in the metabolism of syn oxime appeared to be the bioalteration of the thiocarbamate, $CH_3S\text{-}^{14}C(O)NHCH_3$, to [^{14}C] carbon dioxide.

Discussion

Known routes of pesticide metabolite formation in biological systems are important in attempting to assess the potential hazards associated with the use of a particular compound. With methomyl, formation of carbon dioxide and acetonitrile from the imide carbon indicates that this carbamate yields possibly the most innocuous terminal residues of any pesticide currently in use. Terminal residues of such simple structure must, however, have an intermediate source vastly different than the parent carbamate or its oxime hydrolysis product. Data are presented in the current study which indicate that intermediates of unknown toxicological significance are formed in rats by Beckmann rearrangement of syn and anti methomyl oxime. These products, while in part predictable based on previous work with the compound, have not heretofore been postulated as metabolites of methomyl.

While liberation of carbon dioxide and acetonitrile from the imide carbon was the primary indication that unreported metabolites were involved in methomyl metabolism, additional factors relative to its fate in rats were also instrumental. Of utmost importance was the slow rate of urinary elimination of total radiocarbon from syn [^{14}C=N] methomyl-treated animals. Twenty-four hr after treatment, only 34% of the administered radioactivity was voided by this route. An additional 30% was eliminated in the expired air, but these products, especially acetonitrile, continued to be exhaled for 3 days, demonstrating that their precursors were still in the body. Because 80% of the administered carbamate was hydrolyzed by 6 hr (Table 3), it was apparent that the [^{14}C=N]-labeled residues remaining in the rat were predominantly oximes or their metabolites.

Since [^{14}C] urinary excretion of most carbamates radiolabeled on the alcohol moiety equal that in the urine plus the expired air when the carbamates are radiolabeled on the carbonyl carbon, the low quantity of [^{14}C=N]-labeled metabolites of methomyl in the urine was definitely considered unusual for a member of this group of insecticides. With other carbamates, including aldicarb, the hydrolysis products are conjugated and almost completely excreted from the body within 24 hr. It was assumed, therefore, that a portion of residues in rats treated with [^{14}C=N] methomyl were in a form other than the oxime. Otherwise, far more than 34% of the radiocarbon, presumably oxime conjugates, would have appeared in the urine by 24 hr.

The nature of the persistent precursor to acetonitrile and carbon dioxide was not elucidated, but the evidence suggests that a rather stable complex is formed between a normal body component(s) and the methomyl oximes or their Beckmann rearrangement products. Because the rate of [^{14}C] acetonitrile liberation from anti [^{14}C=N] methomyl was almost the same as for [^{14}C] carbon dioxide from syn [^{14}C=N] methomyl and its oxime (Table 5), the stabilities of the syn and anti complexes appear to be very similar. Therefore, the production of acetonitrile beyond that of carbon dioxide from syn [^{14}C=N] methomyl-treated rats is probably a simple matter of the syn complex being formed before the anti complex.

Complex formation of the type proposed for methomyl metabolism is evident also for thiocarboxime, another thiohydroximic N-methylcarbamate insecticide (Hutson et al., 1971). With syn [$^{14}CH_3{}^{14}$C=N]-labeled material, rats exhaled 5.2% of the dose as [^{14}C] carbon dioxide during the first day, 2.4% on the second day and 0.5% on the third day following oral treatment. Much greater quantities of [^{14}C] acetonitrile (20.9, 10.7, and 4.1% of the dose on each of the first 3 days, respectively) were exhaled, indicating the degree of isomerization to the anti isomer which had occurred.

It should be noted, however, that Beckmann rearrangement of the anti oxime would yield acetonitrile containing both radiocarbons from the parent pesticide, whereas the carbon dioxide from the syn form would contain only one of the radiocarbons. Therefore, the actual amount of [^{14}C] carbon dioxide produced would be double the amounts indicated by the radioactivity collected in the traps. The other radiocarbon would be converted to [^{14}C] methylamine by degradation of the thiocarbamate and excreted via the urine. Considering this stereospecific aspect of acetonitrile and carbon dioxide formation from syn and anti oximes, the amounts of these products formed from thiocarboxime (35.7 and 16.2% of dose after 3 days) were similar to those from methomyl treatments (24.4 and 14.9% of dose after 3 days).

Like methomyl, isomerization of thiocarboxime to the anti form prior to hydrolysis was indicated by treatment of rats with the syn [$^{14}CH_3{}^{14}C=N$] oxime. In this case, the [^{14}C] carbon dioxide expired was about the same amount as when the carbamate was administered. But, the amount of [^{14}C] acetonitrile was reduced to only 7.3% of the dose and this was all collected in the first 24 hr after treatment. It seems that the route of the metabolism of the oximes is dependent almost entirely upon its stereoconfiguration when entering the animal body.

The absence of sulfur oxidation metabolites of methomyl was another facet which gave an indication that previously unreported metabolic products might exist. However, there remains the possibility that the sulfoxide and sulfone derivatives are formed but are unstable. A compound having some structural similarities to the thiohydroximic acids, methylthiotriazine, reportedly was oxidized to the highly unstable sulfoxide form in beans (Knuesli, 1964). Based on a possible similar occurrence with methomyl, the effect of sulfur oxidation on the geometric configurations of thiohydroximic acids was reviewed. It was found that S-methyl benzothiohydroximic acid took on the syn form, while the sulfoxide and sulfone had the anti configuration (Benn et al., 1968).

Applying this to methomyl, it seemed possible that syn oxime could be formed from syn methomyl, and then converted to the anti isomer when oxidation of the sulfur occurred. This way, isomerization after methomyl hydrolysis could occur and subsequently yield both carbon dioxide and methomyl. Because the administration of the syn oxime to rats gave so little acetonitrile, this pathway was not considered adequate to explain the observed results.

Assuming that the thiohydroximate esters, methomyl and thiocarboxime, do not undergo sulfur oxidation as do the alkylthioethers, aldicarb and DS-15647, the explanation may be in the basic chemical nature of the two groups. For example, the alkylthio group of the thiohydroximate esters is bonded to a Sp^2 hybridized carbon which is attached to nitrogen. The effective electronegativity of this carbon atom should be greater than a carbon that is Sp^3 hybridized and bonded to another carbon as is the case with the alkylthioethers. Consequently, the oxidation of the sulfur in the thiohydroximate esters should be more difficult.

In any event, it is now clear that all oxime carbamate insecticides do not undergo the same general type of metabolism. Evidence has been presented showing that in vivo isomerization and Beckmann rearrangement reactions are important in the metabolism of methomyl and are probably applicable to other thiohydroximate esters. These reactions, plus carbamate ester hydrolysis and conjugation of the oximes, result in far more complex routes of bioalteration than previously reported. Moreover, the complexity of their metabolism appears to exceed that of the alkylthioethers which are known to involve primarily defined routes of hydrolysis, sulfur oxidation, and conjugation.

FATE OF CRONETON IN RATS

Croneton, 2-ethylthiomethylphenyl N-methylcarbamate (Bay Hox 1901), is an experimental plant systemic insecticide with excellent aphicidal properties. One of its principal projected uses is the control of these insects on vegetable, fruit and cereal crops. Croneton is highly toxic to aphids, LD_{50} of 5 mg/kg, while the mammalian oral toxicity is rather low, with an LD_{50} of 411 mg/kg to rats (Bayer Information Bull. E.1-715/20 359, 1974).

The potential use of Croneton on food and feed crops necessitates studies of its fate in both plants and animals. This paper reports the results of investigations regarding the insecticide's metabolism in the rat. The fate of Croneton and its sulfoxide (SO) and sulfone (SO_2) analogs was determined in rats following a single oral exposure. Also, animals were exposed to the insecticide as a dietary supplement to determine the degree of residue accumulation which might occur following the more natural avenue of exposure.

Materials and Methods

Chemicals - Croneton was radiolabeled with ^{14}C uniformly on the aromatic ring (7.05 mCi/mmol) and on the carbonyl moiety (5.88 mCi/mmol). The ^{14}C-ring label was supplied by The Chemagro Agricultural Division of Mobay Chemical Corporation and was greater than 99% radiochemically pure. The ^{14}C-carbonyl label was synthesized by the reaction of a 1.5 to 1 ratio of 2-ethylthiomethylphenol and ^{14}C-methylisocyanate in dry benzene. Diacetoxydibutyltin was used as a catalyst.

For use as metabolite standards, 2-ethylsulfinylmethylphenyl N-methylcarbamate (sulfoxide), 2-ethylsulfonylmethylphenyl N-methylcarbamate (sulfone), 2-ethylsulfinylmethylphenol (phenol sulfoxide), and 2-ethylsulfonylmethylphenol (phenol sulfone) were synthesized by the method of Harvey *et al.* (1973), which utilized peracetic acid as the oxidizing agent. Structural confirmation was performed by nuclear magnetic resonance and mass spectrometry. For the NMR spectra, samples were dissolved in deuterochloroform containing 1% tetramethylsilane and measured with a model T-60 spectrometer (Varian Associates). Mass spectra were taken at 60 eV using a Finnegan Model 1015 C quadropole mass spectrometer.

2-Ethylsulfinylmethyl N-methylcarbamate - NMR spectra: δ1.29 (t3H, ethyl CH_3), δ2.60 (q2H, ethyl-CH_2), δ2.87 (d3H, N-CH_3), δ3.90 (d1H, PhCH), δ4.15 (d1H, PhCH), δ5.52 (b1H, NH), and δ7.05-7.55 (m4H, Ph). Mass spectra: m/e 184 (1.9%), 107 (100%), 79 (18.1%), 78 (17.5%), 77 (30.6%), 58 (2.5%), 57 (20.6%), 51 (10.0%).

2-Ethylsulfonylmethyl N-methylcarbamate - NMR spectra: δ1.32 (t3H, ethyl CH_3), δ2.68-3.10 (m5H, ethyl-CH_2 and NCH_3), δ4.28 (s2H, $PhCH_2$), δ5.27 (b1H, NH), and δ7.08-7.68 (m4H, PH). Mass spectra: m/e 200 (5.5%), 107 (100%), 79 (10.0%), 78 (13.5%), 77 (16.5%, 58 (4.5%), 57 (9.0%), 51 (5.0%).

Croneton phenol, the phenol sulfoxide, and the phenol sulfone were also confirmed by NMR and MS. Because a molecular ion (m/e 225) was found for Croneton, the MS of Croneton phenol was easily distinguished. This was not the case for the oxidized material which could only be differentiated from respective phenols by the presence of m/e 58 and 57 ions arising from the carbamate moiety. More positive structural confirmation was obtained by NMR in these cases. In addition, the metabolite standards also were found to be identical to materials supplied by Chemagro.

The purity of the synthesized compounds was determined by thin-layer chromatographic means using the solvent systems given in Table 7. In addition, purity was checked using high pressure liquid chromatography under the following condition: Instrument, two M-6000 pumps plus a model 660 solvent programer (Waters Associates); detector, a model 240

Table 7. SILICA GEL THIN LAYER CHROMATOGRAPHY OF CRONETON AND ITS METABOLITES

Compound	R_f values in solvent systems		
	A	B	C
Croneton sulfoxide	.15	.29	.15
Croneton sulfone	.85	.89	.43
Phenol sulfoxide	.39	.63	.38
Phenol sulfone	.93	.94	.50
Croneton phenol	.98	.97	.71
Croneton	.97	.94	.64

A - Ethyl Acetate

B - 8:2 Acetonitrile-benzene

C - 6:4 Hexane-acetone

UV spectrophotometer detector set at 270 nm (Gilform Instrument Corporation); column - 1/4 in by 30 cm μ Porasil; gradient elution, nonlinear 1% methanol in chloroform to 15% methanol in chloroform for 20 min at 1.0 ml/min. Under these conditions the six Croneton standards were completely resolved.

Treatment - Albino rats (Cox-SD, Laboratory Supply Company) weighing approximately 200 g each were used in all metabolism experiments. In the single oral dose studies, male and female animals were treated with ^{14}C-ring and ^{14}C-carbonyl-labeled Croneton at a dosage level of 0.5 mg/kg. The specific activity of the compounds was adjusted to 2.0 mCi/mmol and delivered to the animals in 0.5 ml of water via a feeding needle. To determine the fate of the oxidation products of Croneton, female rats were similarly treated with the ^{14}C-ring and ^{14}C-carbonyl sulfoxide and sulfone. Sufficient rats were treated so that all data collected represented the results obtained with a minimum of 2 animals.

Croneton-carbonyl-^{14}C and -ring-^{14}C were administered as a dietary supplement to female rats for up to 10 days. The animals were maintained on standard laboratory chow fortified with 6.6 ppm of the insecticide. At this concentration, animals consuming 15 g of feed per day received 0.5 mg/kg/day of the insecticide. Feeding of the Croneton-fortified diet was followed by a normal dietary regime for up to 1 wk. Three rats were used for each predetermined sacrifice time. All animals were acclimated to their surroundings for 1 wk prior to initiating treatment.

Sample Collection and Radioassay - Following treatment with either ^{14}C-carbonyl- or ^{14}C-ring-Croneton, the animals were placed in metabolism cages allowing for separation of urine and feces. Those treated with the ^{14}C-carbonyl material were placed in cages modified for collection of exhaled gases. Urine samples were radioassayed by direct liquid scintillation counting. Feces samples were collected at 24-hr intervals over the 72-hr experimental period and radioassayed by combustion of 0.5 g samples using a Packard Model 306 sample oxidizer. The $^{14}CO_2$ in the exhaled gasses of rats was collected by drawing air from the cages through 300 ml of 1.0 N potassium hydroxide. Samples were then taken for radioassay at hourly intervals for 6 hr post-treatment, then at 3-hr intervals for the next 6 hr and at 12-hr intervals for the remainder of the 72-hr experiment. Radioassay was accomplished by direct scintillation counting of 1.0 ml of the trap solution.

To obtain a measure of the rates of dissipation of ^{14}C-Croneton residues from selected tissues following single oral treatments, 2 animals were sacrificed after 8, 24, 48, and 72 hr. Immediately after sacrifice, a blood sample was taken and the brain, fat, heart, kidney, liver, and a sample of skeletal muscle and skin were excised for radioassay. Radioassay of 0.2-0.5 g tissue samples, with the exception of fat where 50 mg samples were used, was accomplished in the manner as the feces.

Following treatment of female rats with ^{14}C-ring- and ^{14}C-carbonyl-Croneton sulfoxide, or ^{14}C-ring-Croneton-sulfone, the animals were placed in metabolism cages as described above. Urine, feces, and exhaled gases were monitored for radiocarbon for 3 days after treatment. Tissue samples were not analyzed in this series of experiments.

Upon initiation of feeding ^{14}C-Croneton as a dietary supplement, separate collections of urine and feces were made on a daily basis, but no attempt was made to collect the exhaled gases. Sampling of excreta and tissues, and quantitation of the radiocarbon therein, were accomplished using the same techniques as employed in the single dose studies.

Samples collected during the course of the above experiments were stored at -20°C after preliminary radioassay. Periodic analysis of urine and liver over a 6-mo. period showed that the residues were stable at this temperature.

Analysis of Urinary Radiocarbon - Urine was lyophilized to dryness and the solid residue washed with methanol repeatedly until no radioactivity was detected in the wash. This process was quantitative in the recovery of the urinary radiocarbon while leaving much of the interfering urinary components behind. The methanol wash was then concentrated to a volume which allowed direct application to silica gel thin-layer plates. Several solvents systems were used to resolve the radioactive components of the urine (Table 7). In all cases, cochromatography with standards was used to indicate the nature of the metabolites detected.

Analysis of the polar Croneton metabolites in the urine was carried out by reconstituting an appropriate amount of the methanolic fraction with distilled water. Extraction of this aqueous portion with chloroform removed the free carbamates and phenols and the aqueous layer was then adjusted to 0.5 N hydrochloric acid and incubated in a water bath at 37°C. The incubations were conducted in a sealed tube containing an equal volume of chloroform for a period of 6 days. Periodic shaking of the tubes extracted the hydrolyzed polar metabolites into the chloroform layer to prevent further decomposition. The chloroform extracts were combined and analyzed by tlc.

Toxicity Determinations - Oral LD_{50} values were determined for Croneton and its oxygen analogs in both rats and mice. In the case of rats, the techniques of Deichmann and LeBlanc (1943) were used to obtain an approximate LD_{50} only. The animals, 200 g female Cox (SD) albino rats (Laboratory Supply Company), were treated with one of a series of doses which increased by a factor of 1.5 on a mg/kg basis. The compounds were administered in aqueous solution via a feeding needle. For Croneton and its sulfone, 2% Tween-80 was used as the carrier. The approximate LD_{50} values were then calculated on a 24-hr mortality basis. In the case of the mice, LD_{50} values were determined according to techniques of Litchfield and Wilcoxon (1949), using log-dosage/probit analyses. Swiss female albino mice (Laboratory Supply Company) weighing 20 g were treated in groups of 5 with one of six dosage levels which varied over the range of the approximate LD_{50} calculated as above. Again, treatment was by feeding needle and in aqueous solution or Tween-80 as descirbed above. Mortality values were calculated on a 24 hr basis.

Results and Discussion

Excretion - Administration of both ^{14}C-carbonyl- and ^{14}C-ring labeled Croneton to male and female rats as a single oral dose resulted in rapid elimination of the radiocarbon. The pattern of elimination of ^{14}C-carbonyl-labeled Croneton was found to be virtually the same as the elimination pattern of ^{14}C-carbonyl-Croneton sulfoxide. An average of 47% of the dose was exhaled as $^{14}CO_2$ and this occurred predominantly within the first 8 hr after treatment. An additional 41% of the dose was recovered in the urine over a 3-day period while only 7% of the dose was voided in the feces.

Approximately 96% of the ^{14}C-ring-Croneton dose was eliminated from the rats in the urine and about 2% in the feces. Almost identical patterns of excretion were exhibited by ^{14}C-ring-labeled Croneton sulfoxide and sulfone. Like the ^{14}C-carbonyl label, most of the excretion occurred during the first 24 hr following administration, and there was no difference in the rates or quantities of radiocarbon voided by males and females.

Excretion rates of radiocarbon from rats administered ^{14}C-carbonyl- and ^{14}C-ring-Croneton as a dietary supplement were very similar to those observed from the single oral treatments. Approximately 37% of the average daily dose of ^{14}C-carbonyl-Croneton was eliminated in the urine and 7% in the feces. After return to untreated feed, radiocarbon in the urine and feces declined to below 1% of the daily dose in

3 days. The rate of decline was the same for animals fed the treated diet for 7 and 10 days.

Elimination of ^{14}C-ring-Croneton equivalents in the urine averaged 89% of the average daily dose while only 5% was voided in the feces during the treatment period. Like the carbonyl-^{14}C continuous feeding study, a plateau was reached after 3 days on the treated diet. Excretion of radioactive residues declined to below 1% of the average daily dose 3 days after the ^{14}C-Croneton was removed from the diet. Cumulative excretion of the total radioactivity consumed during the 7-day feeding period was 86% at the time the animals were returned to an untreated diet, and 90% 7 days later.

Nature of Urinary Radiocarbon - Results of the analysis of urine from rats which received ^{14}C-Croneton as a single oral dose or in the diet are presented in Table 8. These data represent the results obtained with the ring-^{14}C and carbonyl-^{14}C Croneton. All metabolites identified as carbamates were detected in the urine of rats treated with both ring-^{14}C- and carbonyl-^{14}C-labeled Croneton. Those identified as phenolic hydrolysis products were detected only in the urine of rats treated with Croneton- ring-^{14}C. These data, plus extensive tlc of the metabolites with authentic standards, provided the basis upon which identification of the metabolic products was made.

Qualitatively, the urinary metabolites were sufficiently similar following treatment of the rats with single and multiple doses of the carbamate to suggest that the metabolic pathway for Croneton in rats was not altered by continuous exposure of the animals to the insecticide in the diet. The parent carbamate was not voided in the urine, except possibly in trace amounts which appeared in extracts of acid-treated polar metabolites. While hydrolysis of the carbamate ester was evident, the parent phenol constituted only a small portion of the excreted radiocarbon, and this was almost entirely in the conjugated form.

Table 8. NATURE OF RADIOCARBON ELIMINATED IN THE URINE OF RATS TREATED WITH ^{14}C-CRONETON

Metabolite	Percent of dose/administered as: Single oral dose[a]	Percent of dose/administered as: Dietary supplement[b]
Apolar	36.1	51.4
Croneton sulfoxide	23.0	28.2
Croneton sulfone	3.4	6.9
Parent phenol	0.0	0.4
Sulfoxide phenol	4.5	6.1
Sulfone phenol	2.9	4.5
Unknown A	2.3	5.3
Polar[c]	60.6	34.5
Croneton	1.0	0.9
Croneton sulfone	0.0	4.5
Parent phenol	0.9	7.7
Sulfoxide phenol	18.5	14.4
Sulfone phenol	22.0	3.6
Unknown B[d]	18.2	3.4
Total	96.7	85.9

[a] Combined urine voided over 3-day period following treatment.

[b] Combine urine voided during 7-day feeding period.

[c] Polar metabolites treated with acid to release indicated materials.

[d] Radiocarbon not released by acid treatment.

The remaining metabolites which were identified resulted, at least in part, from sulfur oxidation (Table 8). Croneton sulfoxide was the predominant carbamate metabolite in the urine while the sulfoxide and sulfone phenols were the predominant non-carbamate. Further oxidation of the sulfur resulted in the formation of the sulfone analog which existed as the carbamate and phenol, but in lesser amounts than the sulfoxide derivatives. The majority of the phenolic metabolites were present as conjugates while the carbamate sulfoxide and sulfone were largely in the free form.

In the continuous feeding study, Croneton sulfone was recovered from the acid-treated polar metabolites in quantities equal to 4.5% of the total radiocarbon consumed by the animals. The majority of this metabolite was released immediately upon acidification. Its concentration was similar in urine of rats fed both radioactive Croneton feed preparations, and consistent results were obtained with replicate samples. It is therefore, quite likely that Croneton sulfone was weakly conjugated in rats and was excreted in this form. Croneton sulfoxide appeared in some extracts of the acid-treated polar metabolites, but the quantities were less than the sulfone and its presence was not consistent. It was concluded that the sulfoxide was not conjugated but was in the polar fraction in the free form as a result of incomplete extraction into the apolar phase.

The metabolite designated as unknown A (Table 8) was tentatively identified as 2-ethylsulfonylmethylphenyl N-hydroxymethylcarbamate, or hydroxymethyl Croneton sulfone. Since the metabolite was produced in equal quantities with both the ring-^{14}C and carbonyl-^{14}C Croneton, the ester linkage was assumed to be intact. This was further substantiated by treating the ring-^{14}C-labeled metabolites with 0.5 N sodium hydroxide to yield a product having tlc characteristics identical to sulfone phenol. Reaction of this product with methyl isocyanate gave a compound indistinguishable from Croneton sulfone. These data established that the ring moiety of unknown A was the sulfone phenol and that some modification of the carbamate group also had occurred. N-Methylhydroxylation was proposed since a positive color reaction was evident when the metabolite was treated with chromotropic acid. This test, which indicates the formation of formaldehyde from N-hydroxymethyl compounds, has been used to denote the presence or absence of N-hydroxymethyl metabolites of several carbamate insecticides (Dorough and Casida 1964, Metcalf *et al.* 1968, Black *et al.* 1973).

Unknown A was consistently present in the free form (Table 8) and was sometimes detected in very small amounts in extracts of the acid-treated polar materials. However, its low level and sporatic presence prevented confirmation of its identity as a conjugate.

Treatment of rats with ^{14}C-Croneton sulfoxide as a single oral dose resulted in the same metabolic pattern (Table 9) as with Croneton, per se. The same free metabolites were detected in the urine, and the polar products were of a similar magnitude. There is little doubt that the parent carbamate is very rapidly converted to the sulfoxide form upon entering the animal body by ingestion. Data in Table 9 also confirm that the oxidation of the sulfoxide to the sulfone proceeds at a much slower rate than the initial oxidation. When the sulfone itself was given to the rats, the nature of the metabolites in the urine was as one might predict. All of the free metabolites were sulfone derivatives, and the amounts of polar metabolites were of the same general levels as observed with Croneton and Croneton sulfoxide treatments.

Tissue - Selected tissues were radioassayed for ^{14}C-residues following single oral administration of ^{14}C-carbonyl- and ^{14}C-ring-Croneton. The highest levels of residues were found in tissues of animals treated with the ^{14}C-carbonyl-labeled material. As compared to the ring-^{14}C residues where maximum residues did not exceed 0.05 ppm, they were higher by a factor of 1.4 in the muscle, and by factors of 2.3, 5.8, and 9.1 for the kidney, blood and liver, respectively, when averaged over the 72-hr experiment. Obviously, these increased residues with

Table 9. NATURE OF RADIOCARBON ELIMINATED IN THE URINE OF RATS GIVEN A SINGLE ORAL DOSE OF ^{14}C-CRONETON SULFOXIDE AND CRONETON SULFONE

	Percent of dose[a]	
Metabolites	Croneton sulfoxide	Croneton sulfone
Apolar	35.1	23.3
Croneton sulfoxide	23.8	0.0
Croneton sulfone	4.9	7.8
Sulfone phenol	1.2	10.9
Unknown A	5.2	4.6
Polar	58.3	65.4
Total	83.4	88.7

[a] Combined urine voided over 3-day period following treatment.

the carbonyl-^{14}C Croneton treatment were derived only from the carbamate portion of the molecule. This would suggest that the radioactive carbon liberated upon hydrolysis was incorporated in natural tissue components.

Because of the very rapid metabolism and excretion of Croneton, and the low level of ring-^{14}C residues in rat tissue 8 hr after treatment, an experiment was conducted to determine if peak residue levels occurred prior to the 8-hr sampling period. Female rats were treated orally with 5 x 10^5 dpm (7.3 μg) of the ^{14}C-ring-labeled Croneton and then an animal sacrificed at each 30 min interval for 8 hr. Samples of the blood, liver, kidney, and muscle were collected and radioassayed. The point at which maximum ^{14}C-ring-Croneton equivalents occurred in the tissues ranged between 2 and 2.5 hr after administration of the single oral dose, with the blood and muscle showing the earlier peak. These data clearly demonstrated the extreme rapidity with which Croneton and/or its metabolites are absorbed from the gut, distributed throughout the body and then eliminated. With such efficient metabolism and excretion, there appears to be little potential for residue accumulation.

Analysis of tissue samples collected from rats maintained on a diet containing 6.6 ppm of either ^{14}C-carbonyl- or ^{14}C-ring-Croneton substantiated the results seen in the single oral dose studies. In those rats fed the ^{14}C-carbonyl-label material, the majority of the tissues increased in Croneton equivalents as the time of exposure increased. However, the rate of increase was very slow after the second day of exposure of the animals to the treated diet. At the end of 7 days the levels were 1.18, 0.29, 0.24, 0.10, 0.18, and 0.06 ppm for the liver, kidney, blood, muscle, brain, and fat, respectively. Those animals fed the ^{14}C-ring-labeled compound showed much lower ^{14}C-residue levels in the same tissues and they increased very little after the second day of feeding.

When the animals were returned to an untreated diet, ^{14}C-ring-residues in the tissues declined at a much faster rate than the ^{14}C-carbonyl-labeled residues. This pattern of dissipation would be expected if the latter residues resulted in part from the incorporation of the radioactive carbon into normal tissue constituents. ^{14}C-Carbonyl-Croneton equivalents were between 0.04 and 0.11 ppm 7 days after removal from treatment, while the ^{14}C-ring-labeled residues had decreased below the 0.01 level after only 2 days.

Because of the low levels of Croneton-ring-^{14}C residues in the tissues, no attempt was made to characterize the radiocarbon therein. However, livers of rats fed Croneton-carbonyl-^{14}C in the diet were evaluated as to extraction characteristics, but their levels were too low for evidence required for identification to be obtained. Homogenization of livers of rats fed 1, 2, 4, and 7 days with a 3:1 acetonitrile-water mixture removed approximately 30% of the ^{14}C-residues in each of the samples. Treatment of the tissue solids with 1.0 N hydrochloric acid at 80°C for 1 hr released an additional 44% of the residues; these could not be extracted from the aqueous phase with chloroform. The acetonitrile-water extract of the livers also was extracted with chloroform and, in this case, about two-thirds of the radiocarbon partitioned into the organo-solvent layer. Treatment of the aqueous layer with 1.0 N hydrochloric acid for 1 hr at 80°C did not convert any of the radioactivity into chloroform-extractable materials.

Thin-layer chromatography of the chloroform extract of the liver homogenates was unsuccessful because of the small amount of radioactivity and the large quantities of oils and other interfering materials present. Treatment of the chloroform-extractables with a coagulation solution (1.25 g ammonium chloride and 35 ml of phosphoric acid diluted to 1 liter) resulted in all of the radioactivity being removed from the solution along with the precipitate. Thus, none of the ^{14}C-residues in the liver of rats fed ^{14}C-carbonyl-Croneton exhibited extraction and/or partitioning characteristics typical of free or acid-released Croneton-type metabolites.

Toxicity - An an indicator of the biological significance of Croneton and its metabolites, the toxicities of the parent carbamate and its oxidized sulfur analogs were determined in both rats and mice (Table 10). The mice were much more sensitive to the carbamates than the rats. In mice, the toxicity of Croneton and its sulfoxide was not statistically different (71 and 59 mg/kg, $p<.05$) while Croneton sulfone was only about one-fourth as toxic. While less toxic to rats, the relative toxicities were approximately the same in rats as in mice.

Table 10. TOXICITIES OF CRONETON AND ITS OXIDIZED SULFUR ANALOGS TO RATS AND MICE[a]

	APPROXIMATE LD_{50} (mg/kg)[b]		LD_{50} (mg/kg)[c]
Compound	Rats	Mice	Mice
Croneton	338	150	71
Croneton sulfoxide	150	67	59
Croneton sulfone	506	225	282

[a] Twenty-four hr LD_{50} following single oral dose.

[b] Determined by the approximation method of Deichmann and LeBlanc (1943).

[c] Determined by log-dosage/probit analyses of Litchfield and Wilcoxon (1949).

FATE OF CRONETON IN LARGE ANIMALS

Croneton (2-ethylthiomethylphenyl-N-methylcarbamate) is an experimental insecticide which may be used on crops constituting a portion of the diets of livestock. Therefore, it is important to determine the fate of the insecticide in these animals so that the potential for the transfer of residues to the diet of man can be estimated. In the present study, results of the fate of a single oral dose of radioactive Croneton to a lactating Holstein cow, a male Yorkshire pig, and to laying White Leghorn hens are presented. Experiments with the hens were extended to include daily treatments with Croneton for 7 days so that the effect of multiple exposure on metabolism and disposition of the carbamate could be evaluated. The fate of Croneton in rats following single and multiple treatments of the insecticide has been determined in our laboratory and the results previously reported (Nye *et al.* 1976).

Materials and Methods

Chemicals - To determine the fate of Croneton in the animals, [^{14}C]-ring-labeled insecticide was used (specific activity, 2.0 mCi/mmol). This was supplied by the Chemagro Corporation and had a radiochemical purity of greater than 99% as determined by thin-layer chromatographic and autoradiographic techniques. To aid in metabolite identification by cochromatography, the following compounds were synthesized by methods previously described (Nye *et al.* 1976); 2-ethylsulfinylmethylphenyl N-methylcarbamate (Croneton sulfoxide), 2-ethylsulfonylmethylphenyl N-methylcarbamate (Croneton sulfone), 2-ethylthiomethylphenol (Croneton phenol), 2-ethylsulfinylmethylphenol (phenol sulfoxide), and 2-ethylsulfonylmethylphenol (phenol sulfone).

Treatment and Sampling - Cow and pig - Both the lactating Holstein cow (weighing approximately 500 kg with milk production of 22 kg/day) and the male Yorkshire pig (approximate weight of 50 kg) were acclimated to the experimental ambience for 3 days prior to treatment. Croneton was administered at a level of 0.5 mg/kg in a gelatin capsule containing feed grain. Following treatment, the cow was placed in a metabolism stall and separation of urine from feces was accomplished by an intraurethral catheter implanted prior to treatment. The swine was also placed in a metabolism stall which allowed for separate collection of urine and feces.

Urine eliminated by the cow was sampled on an hourly basis for the first 6 hr after treatment, at 2 hr intervals for the next 6 hr, and at 4 hr intervals for the next 12 hr. Urine eliminated by the pig was sampled periodically during the 24 hr experiment and collection was dependent on the intervals of urination. Radioassay of the urine samples was by direct liquid scintillation counting of 0.5 ml samples. Feces samples were collected from both animals periodically and radioassayed by combustion of 1.0 g samples using a Packard Model 306 Sample Oxidizer.

In addition to the urine and feces samples, blood (5.0 ml) and milk from each quarter (5-10 ml) were taken from the cow at the same time intervals as the urine samples. At 6 and 21 hr after treatment, the animal was milked in a normal manner using a mechanical milker. The blood samples, taken from the abdominal vein, were immediately heparinized and later radioassayed by combustion of 0.5 ml samples as described above.

At 24 hr post-treatment, the cow and pig were slaughtered and representative tissue samples immediately frozen for later analysis. Radioassay of 0.5 g samples (0.05 g in the case of adipose tissue) was accomplished by combustion in the same manner as for the blood and feces samples.

Hens - In the first study, eight hens were treated with a single oral dose of Croneton at a rate of 0.5 mg/kg (sp. act., 2.0 mCi/mmol). The dose was prepared by adding the ring labeled insecticide to gelatin capsules containing a small amount of laying mash. Following treatment, the birds were placed in individual cages and eggs and excreta were collected at 12-hr intervals for 3 days. No attempt was made to separate urine and feces. At 8 hr, and at 1, 2, and 3-day intervals after treatment, two birds were sacrificed and tissue samples collected.

In the second study, 10 birds were given the capsules containing Croneton on a twice-daily basis at about 12-hr intervals for 7 days. The specific activity of the ring [^{14}C] material was 4.0 mCi/mmol to facilitate tissue radiocarbon analysis which was difficult at the dosage level used in the first study. Eggs and excreta were collected prior to treatment and on a daily basis after treatment was initiated. On the 4th day of treatment and prior to administration of the second daily dose, one bird was sacrificed and tissues taken for radioassay. At 4 hr following the final treatment, four birds were sacrificed in an attempt to achieve maximum tissue [^{14}C]-ring Croneton equivalents. Two birds were sacrificed 1 day after the final treatment and one bird sacrificed at 2, 4, and 7 days after the last capsule was given to establish rates of dissipation of [^{14}C]-ring Croneton residues.

In all experiments, the birds were maintained on a 12-hr light and 12-hr dark photo period and were given laying mash and water *ad libitum*. All samples were stored in the freezer until analyzed.

Tissue samples and the excreta were radioassayed by combustion as previously described. The eggs also were analyzed by combustion, but they were first divided into shell, yolk and whites, and analyses were performed using 0.2-g samples.

Metabolite Identification - Excreta - Aliquots of urine from the cow and swine, or excreta from the hens were lyophilized prior to the determination of the nature of the radiocarbon content. The dry solid residues were then repeatedly washed with methanol which removed greater than 95% of the [^{14}C]-ring Croneton equivalents while leaving a large portion of the interfering components behind. The methanolic fraction was concentrated to a volume suitable for direct application to silica gel thin layer chromatographic plates. Two basic solvent systems were used to develop the tlc plates; a 6:5 hexane-acetone mixture for one-dimensional chromatography, and a two-dimensional system using 6:5 hexane-acetone in one direction and 10:1 ethyl acetate-hexane in the second direction. The first system was used as a preparative method for analysis of free metabolites, while the two-dimensional system was used for attempted co-chromatography of metabolites with known standards. In addition, a benzene-acetonitrile solvents system (of varying composition) was used to confirm the results of the two-dimensional system.

The polar metabolites remaining at the origin in the above systems were subjected to analysis by incubation with 0.5 N hydrochloric acid. The acidified samples were incubated for 6 days at 45°C and the acid-released metabolites were removed by extraction with chloroform at 24-hr intervals.

In addition to acid hydrolysis, the polar metabolites were incubated with β-glucuronidase (type V-A, Sigma Chemical Company) and aryl sulfatase (type III, Sigma Chemical Company). Fifty units of β-glucuronidase or 10 units of aryl sulfatase were added to the extracted metabolites in acetic acid-sodium acetate buffer (0.1 M, pH 5.6) and the mixture incubated for 24 hr at 37°C. The preparations were then extracted with three equal volumes of chloroform, and the chloroform concentrated and applied to tlc.

Milk - To determine the nature of the radiocarbon residues in milk, 100 ml of the 6-hr sample were mixed with 200 ml of acetone to precipitate the solids. The solids were separated by filtration and the filtrate extracted three times (100 ml, 75 ml, and 75 ml) with chloroform. The combined chloroform extracts were concentrated and analyzed by tlc using the 6:5 hexane-acetone solvents system.

The aqueous phase of the milk was acidified to 1.0 N with hydrochloric acid and heated for 1 hr at 80°C. Radiocarbon released by acid treatment was extracted into chloroform and analyzed by tlc.

Eggs - Eggs collected during the continuous feeding study were analyzed by homogenizing the combined whites and yolks in acetonitrile for 3 min in a Lourdes homogenizer. The solids were removed by filtration and twice again homogenized with acetonitrile. The acetonitrile filtrates were combined and 100 ml of 0.5 N hydrochloric acid were added. This mixture was extracted with four 60-ml aliquots of chloroform, the chloroform dried over anhydrous sodium sulfate, and then evaporated to dryness. Several washings of the evaporating flask with a precipitation solution (1.25 g of ammonium chloride and 25 ml of phosphoric acid in sufficient water to make a final volume of one liter) were used to transfer the residue to a separatory funnel. The solution was extracted as before with chloroform, the solvent dried with sodium sulfate and then concentrated for application to tlc plates.

The water resulting from the first chloroform extract was acidified to 1.0 N with hydrochloric acid and heated at 80°C for 1 hr. This solution was extracted with chloroform. Solids remaining after homogenization were similarly treated with a 1.0 N hydrochloric acid solution for 1 hr at 80°C and the solution filtered. The filtrate was extracted with chloroform and both phases were radioassayed.

Results

Cow and Pig - Uptake and excretion - Maximum concentrations of [^{14}C]-ring Croneton equivalents in cow blood, 0.32 ppm, occurred 3 hr after treatment. The appearance of radiocarbon residues in the milk was somewhat slower with the maximum concentration of 0.15 ppm observed 4 hr after administration of the capsule. These results are similar to those observed in rats where blood and tissue [^{14}C]-Croneton equivalents were highest after 2.0 to 2.5 hr (Nye *et al.* 1976). [^{14}C]-Croneton equivalents in the urine reached a maximum concentration 3 hr after administration. Cumulative excretion of [^{14}C] Croneton equivalents via the urine was greater than 90% of the dose by 12 hr. After 24 hr, 97.8% of the dose had been voided in the urine while less than 1% was in the feces. In the pig, elimination was also principally via the urine, with 80% of the dose excreted within the first 12 hr. After 24 hr, 90% of the dose had been eliminated in the urine and 5.1% was present in the feces.

Tissues - Following slaughter of the swine 24 hr after treatment, combustion analysis of various tissues revealed no detectable radioactive residues. Essentially the same findings were obtained with the bovine tissues. Of 18 different tissues analyzed, only the kidney (0.016 ppm), liver (0.017 ppm), and skin (0.05 ppm) contained detectable levels of radiocarbon.

Urinary metabolites - There were several quantitative differences in the nature of the Croneton metabolites in the cow and pig urine (Table 11). The cow, for example, excreted Croneton principally as water soluble metabolites, 86.9% of the dose, with the phenol sulfoxide and phenol sulfone being the only significant free metabolites. The swine, on the other hand, eliminated the [^{14}C]-ring-Croneton equivalents to a lesser extent as water soluble metabolites (53.3% of the dose). Of the total radiocarbon in the pig urine, 26% was as free Croneton phenol, 9% as free phenol sulfoxide and 6% as free phenol sulfone.

Analysis of the polar urinary [^{14}C]-components, those remaining at the tlc origin, was attempted by acid hydrolysis. In the case of urine collected from the cow, incubation at 45°C for 6 days in 0.5 N HCl or at 80°C for 2 hr at 4.0 N HCl, gave identical results. Only 25% of the polar metabolites was recovered by chloroform extraction. Nearly one-half of the radiocarbon released with acid was identified as the phenol sulfoxide. The remainder consisted of Croneton sulfoxide, phenol sulfone, Croneton, phenol, and unknown II. The latter metabolite had the same characteristics as a metabolite formed in rats (unknown A, Nye *et al.* 1976) and in chickens (unknown II). Incubation of the polar metabolites with β-glucuronidase was more successful at releasing the [^{14}C]-Croneton equivalents from the polar form than treatment with acid

Table 11. NATURE OF RADIOCARBON IN 0-12 HOUR URINE OF COW AND PIG TREATED WITH A SINGLE ORAL DOSE, 0.5 MG/KG, [^{14}C] RING-CRONETON.

Metabolites[a] F - Free A - Acid-released G - Glucuronidase-released S - Sulfatase-released		% of total [^{14}C] in sample	
		Cow	Pig
Croneton	- A	0.0	9.0
Croneton sulfoxide	- F	0.5	0.0
	- A	0.9	0.0
	- G	0.0	2.1
	- S	0.0	2.0
Croneton phenol	- F	0.0	25.5
Phenol sulfoxide	- F	8.7	9.1
	- A	12.5	12.2
	- G	37.9	12.1
	- S	4.8	18.0
Phenol sulfone	- F	2.4	6.0
	- A	1.0	1.1
	- G	6.3	4.3
	- S	1.8	4.3
Unknown II	- A	7.5	0.0
Polar unknown[b]	- A	66.5	33.6
	- G	44.2	40.9
	- S	81.8	35.1

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[a] After removing the free metabolites, the polar radiocarbon was separated into 3 portions and individually treated with acid, glucuronidase or sulfatase. Therefore, the released metabolites are not cumulative.

[b] Radiocarbon remaining in water phase after indicated treatment.

(Table 11). Tlc analysis revealed that conjugated phenol sulfoxide constituted 37.9% of the radiocarbon in the urine and the phenol sulfone, 6.3%. Aryl sulfatase treatment released only 4.7% and 1.7% as the phenol sulfoxide and phenol sulfone, respectively. In neither case did the enzyme treatment release unknown II.

Only 33.6% of the Croneton equivalents in the swine urine remained as water soluble components after treatment with acid (Table 11). About 9% of the total [^{14}C] in the urine was released as phenol sulfoxide, while only 1% was released as the phenol sulfone. In addition, 9% was released as a product with an R_f identical to Croneton. Unknown II was not observed in the acid released fraction of the swine urine.

Enzymatic hydrolysis of the polar origin Croneton equivalents was only slightly more effective than treatment with acid, and the radiocarbon released was nearly equal for glucuronidase and sulfatase. As was the case for the cow urine, the major component released by enzymatic hydrolysis was the phenol sulfoxide. After glucuronidase treatment, 41% of the radioactivity in the urine remained in the water phase while 35% remained after treatment with sulfatase.

It appears that the differences that occurred between the two animals with regard to the fate of Croneton was their ability to oxidize the parent carbamate as well as to conjugate the phenolic metabolites of Croneton. The cow had the greater ability to perform these functions, while the swine had a somewhat restrictive ability to form conjugates of the products resulting from hydrolysis of the carbamate. It is important, however, that this did not limit the swine's ability to eliminate the ingested compound.

Milk metabolites - While no appreciable radiocarbon was detectable in the milk 21 hr following administration of the bolus, analysis of the metabolites in the 6-hr sample (0.3% of the dose) gave a good indication of the nature of the products excreted in milk. Of the total radiocarbon in the milk, 67.3% was organosoluble and identified primarily as the sulfoxide and sulfone of the carbamate (Table 12). Small amounts of the phenol sulfoxide and sulfone were also present in the free form.

Acid hydrolysis of the water soluble fraction yielded organo extractables primarily as Croneton sulfoxide and phenol sulfoxide. Other minor products identified were Croneton phenol, phenol sulfone, and Croneton sulfone. An unknown metabolite (II), tentatively identified as N-hydroxymethyl Croneton sulfone, was detected in milk analyzed shortly after collecting the sample, but was not observed in samples stored after 9 months. Other metabolites were stable in the frozen milk. Initially, unknown II represented 0.9% of the total radiocarbon in the milk or 1.2 ppb [^{14}C] Croneton equivalents. Water soluble materials which were not acid labile constituted 17.3% of the [^{14}C] residues in the milk, while only 3.1% was associated with the solids precipitated by the addition of acetone.

Hens - Excretion and distribution of residues - Elimination of the single oral dose of Croneton to White Leghorn laying hens was very rapid with greater than 80% of the dose voided in the excreta within 24 hr, and greater than 90% after 3 days. A somewhat different pattern was observed in the 7-day study. When the insecticide was administered on a continuous basis, elimination via the excreta was somewhat low for the first 2 days following initiation of treatment with 60% of the cumulative dose being excreted; however, 90.2% of the radiocarbon consumed over the 7-day period had been eliminated when treatment was terminated.

Results of the radioassay of selected tissue samples collected after administration of both a single oral dose, or continuous treatments of the insecticide are given in Table 13. At 8 hr after the single dose, slight tissue residues were present but dissipation of Croneton equivalents from the tissue was very rapid and no detectable [^{14}C]-equivalents were found 24 hr post-administration.

Table 12. NATURE OF [^{14}C] CRONETON EQUIVALENTS IN MILK OF COW TREATED WITH A SINGLE ORAL DOSE, 0.5 MG/KG, OF [^{14}C] RING-CRONETON.

Metabolites or fraction	PPB and % distribution/6-hr milk sample	
	PPB	%
Free	86.1	67.3
Croneton sulfoxide	50.6	39.6
Croneton sulfone	24.7	19.3
Phenol sulfoxide	7.2	5.6
Phenol sulfone	3.6	2.8
Water soluble	37.8	29.6
Acid-released	15.7	12.3
Croneton sulfoxide	7.2	5.6
Croneton sulfone	0.4	0.3
Croneton phenol	0.6	0.5
Phenol sulfoxide	4.8	3.8
Phenol sulfone	1.5	1.2
Unknown II	1.2	0.9
Remaining in water	22.1	17.3
Milk Solids	4.0	3.1
Total	127.9	100.0

Table 13. [^{14}C] RING CRONETON EQUIVALENTS IN TISSUES OF HENS ADMINISTERED THE INSECTICIDE AS A SINGLE ORAL DOSE OR AS TWICE DAILY TREATMENTS FOR 7 DAYS (0.5 MG/KG PER DOSE).

	Ppm Croneton equivalents						
		Daily treatment					
	oral dose[a]	On treatment[b]		Off treatment			
Tissue	8 hr	4 days	7 days	1 day	2 days	4 days	7 days
Blood	0.053	0.016	0.099	0.010	0.009	ND	ND
Brain	0.028	0.001	0.039	ND	ND	ND	ND
Fat	ND	ND	0.019	ND	ND	ND	ND
Heart	ND	0.005	0.072	0.004	0.005	ND	ND
Kidney	0.062	0.016	0.324	0.022	0.018	0.007	0.006
Liver	0.035	0.025	0.180	0.044	0.034	0.009	0.011
Muscle	0.039	ND	0.069	0.004	0.006	0.001	0.002
Skin	0.022	0.013	0.075	0.007	0.012	0.006	0.004

[a] Tissues were analyzed at 1, 2, and 3 days after treatment, but did not contain detectable levels of residues.

[b] Higher residues at 7 vs. 4 days was due largely to the fact that the 7-day birds were sacrificed 3-4 hr after last treatment while 4-day birds were sacrificed 8 hr after last treatment.

Accumulation of insecticide equivalents in the tissues was minimal as indicated by the low levels found in isolated tissues 4 days after initiation of the daily treatments. Although [^{14}C]-residues were higher in the tissues at 7 days, this was due largely to the early sampling time after the last dose was administered (4 hr) rather than from accumulation. With the 4-day samples, the hens were sacrificed 8 hr after the prior treatment. [^{14}C]-Residues in all tissues dissipated very rapidly and most were below the level of sensitivity (.005 ppm) within 2 days after treatment was terminated.

Eggs collected following the single oral treatment contained no [^{14}C]-ring Croneton equivalents in either shell, white, or yolk. [^{14}C]-Residues in eggs collected during the continuous feeding study were evident 2 days after initiation of treatment. The shell showed the lowest level of insecticide residues, reaching a high of 16.1 ppb on the third day. Residues in the white and yolk were nearly equal for the first 4 days of treatments and averaged 38 ppb. While residues in the white increased very little thereafter, those in the yolk continued to rise and were on the order of 70 ppb on the last day of treatment.

A large variation existed between the eggs sampled at any given time during the continuous feeding experiment. The location of residues within the eggs appeared to be dependent on the stage of egg development at the time the dose was administered and certain eggs within each sampling time were found to contain the entire residue within either the white or the yolk.

Nature of metabolites - The nature of the [^{14}C]-ring-Croneton equivalents found in the excreta is given in Table 14. The differences in metabolism that existed between application by single oral dose or continuous feeding occurred in two areas. First, the quantity of polar materials in the excreta of the single-dosed hens was lower than that observed in the continuous feeding experiment. This difference was minimized after acid hydrolysis, as similar amounts of [^{14}C]-ring Croneton equivalents remained

Table 14. NATURE OF RADIOCARBON IN EXCRETA OF HENS TREATED WITH [^{14}C]-RING LABELED CRONETON

Metabolites F = Free A = Acid-released		% of total [^{14}C] in sample			
		Single oral dose	Continuous treatments/days		
			1	4	7
Croneton sulfoxide	- F	0	2.7	1.3	1.7
	- A	0	0	1.2	0
Croneton sulfone	- F	0	0.9	0.9	0.7
Croneton phenol	- F	2.9	1.3	2.6	2.5
Phenol sulfoxide	- F	23.9	2.6	5.1	6.0
	- A	1.0	9.8	2.6	3.4
Phenol sulfone	- F	36.5	13.3	29.3	20.2
	- A	1.6	15.1	12.3	14.1
Unknowns	- F	0	12.1	9.6	10.6
	- A	7.1	7.2	9.4	13.3
Unknown polar[a]		27.0	35.0	25.7	27.4

[a] Radiocarbon remaining in water phase after acid treatment.

as unknown polar products in both studies. A second difference was in the nature of the radiocarbon which existed as free metabolites, and which could be released with acid. Like the single oral dose experiment, the major metabolites in the excreta of the multiple-dosed hens were the phenol sulfoxide and phenol sulfone. However, conjugation of these products was much greater in the continuous feeding study. Some small amounts of the sulfoxide and sulfone of the carbamate were also found in the continuous feeding study, but not in the single dose experiment.

Major differences in the quantity of unknown metabolites were found in the two studies (Table 14). Unknown II (10.6% of the dose) has the same R_f as an unknown isolated and tentatively identified as the N-hydroxymethyl Croneton sulfone in previous rat studies (Nye *et al.* 1976). As found in the earlier study, unknown II in the chicken excreta was unstable, and was converted to the sulfone phenol under acid conditions. Several other unknowns were also released by acid hydrolysis but were not identified because of their low individual quantity. Treatment of the water soluble metabolites with β-glucuronidase and aryl sulfatase did not release any [^{14}C]-ring Croneton equivalents into the chloroform extract.

The nature of [^{14}C]- residues in the tissues was not evaluated. Total radiocarbon was either too low in tissues of bulk, or the sample too small to yield sufficient [^{14}C] to follow through the various fractions resulting from extraction and partitioning. This was not the case with eggs (Table 15).

A majority of the [^{14}C]-residues in the eggs was organosoluble and, as was the case for metabolites in the excreta, those in the eggs were principally sulfoxidation products of Croneton phenol. Combined, the sulfoxide and sulfone of the phenol accounted for 71.4, 75.5, and 78.3% of the total residues in the eggs collected on day 3, 6, and 7. Small amounts of the oxidized carbamates and unknown products were also de-

Table 15. NATURE OF $[^{14}C]$ CRONETON EQUIVALENTS IN EGGS OF HENS DOSED EVERY 12 HR WITH 0.5 MG/KG $[^{14}C]$ RING CRONETON FOR 7 DAYS.

	PPB and % distribution in eggs laid on day			
Metabolites	3	6	7	
or fractions	PPB	PPB	PPB	%
Free	41.82	48.99	63.34	88.5
Croneton sulfoxide	2.01	2.78	2.77	3.9
Croneton sulfone	3.68	3.14	2.89	4.0
Phenol sulfoxide	9.79	11.05	14.04	19.6
Phenol sulfone	24.00	30.89	42.04	58.7
Unknowns	2.34	1.13	2.60	2.3
Water Soluble	2.25	2.70	3.35	4.7
Acid-released	0.57	0.69	0.85	1.2
Remaining in water	1.68	2.01	2.50	3.5
Unextractables	3.27	3.92	4.86	6.8
Acid-released	0.50	0.63	.79	1.1
Remaining in solids	2.77	3.29	4.07	5.7
Total	47.34	55.61	71.55	100.0

tected. [^{14}C]-Residues in the water phase and solid fraction contained a combined total of 5 to 10% of the total radiocarbon in the eggs.

Discussion

The carbamate insecticides are generally characterized as being efficiently metabolized and rapidly excreted by animals (Kuhr and Dorough, 1976). This very desirable feature has been of paramount importance in keeping alive the search for carbamates of commercial value during the past ten years. While progress was slow in the 1960's, several carbamates have more recently achieved commercial success in the United States and in other countries, and this success has stimulated greater research efforts on this group of insecticides.

Croneton, a Bayer product designated by the company initially as BAY HOX 1901, is one of the newer carbamate insecticides and one whose metabolism in animals has been studied extensively in our laboratory. Results obtained in the present study with the cow, pig, and hen are similar to those found with the rat (Nye *et al.* 1976). However, species differences do exist which are both of interest and of possible practical significance. Differences, as well as similarities, can best be demonstrated by considering the radiocarbon in the excreta of animals treated with a single oral dose of [^{14}C]-ring Croneton (Table 16).

It is evident that the efficiency of excretion of [^{14}C] Croneton equivalents over a 24-hr period following treatment varied little among the species tested. In every case, 90% or more of the dose had been excreted. A major difference in the nature of the excreted radiocarbon was that the rat urine contained rather large quantities, 24% of the dose, of free carbamates, mostly Croneton sulfoxide. None of the large animals eliminated any free metabolite with the carbamate ester linkage intact. The pig and hen, however, did excrete much of the dose as free phenolic analogs of Croneton. They were minor constituents in cow and rat urine. Excretion of the free metabolites by the pig and hen, and

Table 16. COMPARATIVE NATURE OF RESIDUES IN THE URINE OF ANIMALS TREATED WITH A SINGLE ORAL DOSE OF [^{14}C]-RING LABELED CRONETON.

Metabolites	% of dose as indicated metabolite			
	Rat	Cow	Pig	Hen[a]
Free				
Croneton sulfoxide	22	0	0	0
Croneton sulfone	2	0	0	0
Croneton phenol	0	0	26	3
Phenol sulfoxide	3	9	8	23
Phenol sulfone	3	2	6	34
Water solubles	60	87	50	31
Total	90	98	90	91

[a] Total excreta collected.

the corresponding low levels of water soluble products demonstrated that conjugation is not always necessary for efficient elimination of phenolic metabolites of the carbamates.

Because of the very efficient excretion of Croneton by the animals, residues in the tissues were either absent or too low in quantity for characterization. Examination of the milk and eggs (from hens treated daily for 7 days) showed that these products contained [^{14}C] residues quite different in nature (Table 17). While a majority of the residues in each substrate was of an organosoluble nature, these free metabolites in milk were comprised largely of Croneton sulfoxide and Croneton sulfone, but were almost entirely Croneton phenol in eggs. Levels of bound, or unextractable, residues were exceedingly small in both milk and eggs.

One could continue to dissect the data generated in the animal studies with Croneton, and continue to reveal interesting similarities and differences among species. However, the most germane points have been emphasized and further comparisons can best be made individually by those with specific points of interest. Generally, it may be concluded that the metabolism of Croneton in animals is quite typical of that expected of an N-methylcarbamate containing an ethylthiomethyl substituent on a phenyl ring. While different animal species exhibited metabolic differences which could be toxicologically significant, the rapid rate of excretion of free as well as conjugated Croneton metabolites lessened their potential significance considerably. Thus, the production of free metabolites of sufficient polarity to effect urinary elimination without further metabolism (for example, Croneton sulfoxide in rats) is one characteristic which distinguishes Croneton from most other carbamate insecticides.

Table 17. [^{14}C] CRONETON EQUIVALENTS IN MILK AND EGGS

Metabolites	% of [^{14}C] in sample Milk[a]	 Eggs[b]
Free	67	88
Croneton sulfoxide	39	4
Croneton sulfone	19	4
Phenols	9	79
Unknowns	0	1
Water solubles	30	5
Unextractables	3	7

[a] Milk sample collected 6 hr after single oral dose, 0.5 mg/kg, of [^{14}C] ring Croneton.

[b] Eggs samples on 7th day of treatment of hens; 0.5 mg/kg twice daily.

FATE OF INSECTICIDES ADMINISTERED ENDOTRACHEALLY TO RATS

The evaluation of the fate of insecticides in animals has been classically determined after oral, dermal, and/or intraperitoneal administration of the compounds. Little information is available on the metabolic fate of these chemicals when inhaled, although inhalation toxicity studies are commonly conducted on products being developed for commercial use. The chemical and biological fate of inhaled insecticides could greatly influence their toxicity to animals, and a knowledge of their fate would be useful in estimating the potential hazards of these toxicants following exposure by inhalation.

In the present study, insecticides were quantitatively administered as aerosols to the lungs of rats via the trachea. The relative rates of absorption by the blood and the fate of the insecticides in the animals were determined.

Materials and Methods

Insecticides - Four ^{14}C-labeled insecticides were selected for this study: carbaryl [1-naphthyl-^{14}C N-methylcarbamate (6,600 dpm/μg) and the carbonyl-^{14}C form (4,400 dpm/μg)], leptophos [O-methyl O-4-bromo-2,5-dichlorophenyl-^{14}C phenylphosphonothioate (7,800 dpm/μg) and O-methyl O-4-bromo-2,5-dichlorophenyl phenyl-^{14}C-phosphonothioate (8,000 dpm/μg)], parathion [O,O-diethyl O-p-nitrophenyl-2,5-^{14}C phosphorothioate (13,600 dpm/μg) and the diethyl-^{14}C form (13,000 dpm/μg)] and chlordane-^{14}C (11,500 dpm/μg).

Treatment - Female Sprague-Dawley rats weighing approximately 200 g each were surgically prepared for treatment using techniques previously described (Atallah and Dorough 1975, Burton and Shanker 1974). Each animal was anesthetized with 13 mg of sodium pentobarbital, IP, which immobilized them for the duration of the experiment. A cannula was inserted between the fourth and fifth tracheal ring below the thyroid cartilage through which the insecticide solution was administered.

The radiolabeled insecticide in 20 µl of ethanol was delivered to the lungs as an aerosol by depressing the plungers of the 50-µl and 5-ml syringe simultaneously. Thus, the insecticidal aerosol was delivered to the lungs in a total air volume of 5 ml, held for 15 sec to achieve maximum retention, and then the cannula leading from the trachea connected to a carbon dioxide trap (2:1 solution of 2-methoxyethanol and 2-aminoethanol). Blood samples were taken from the tail periodically for one hour and the animals then sacrificed and tissue samples collected for analysis.

Blood and Tissue Analysis - The blood, 0.2-0.4 ml, and tissue samples, 400-800 mg, were placed in quartz vessels and combusted in a Beckman Biological Materials Oxidizer. The radiocarbon generated as $^{14}CO_2$ during combustion was trapped and an aliquot of the solution radioassayed by liquid scintillation counting (Packard Model 3380/544).

Excretion Studies - Carbaryl-ring-^{14}C, leptophos-phenoxy-^{14}C, parathion-phenyl-^{14}C, and chlordane-^{14}C were administered to female rats in the manner described above. In this case, however, ether was used as the anesthetic and the incision closed immediately after administration of the insecticides. The animals were placed in plexaglass metabolism cages and the excreta monitored periodically for radiocarbon until excretion of radioactivity had ceased.

Results and Discussion

Administration of the dose to the rats via the trachea resulted in quantitative retention of the inhaled insecticides. None of the intact insecticides was detected in the exhaled air. In fact, the only radiocarbon exhaled at all was ^{14}C-carbon dioxide, 2.5% of the applied dose, from animals treated with ^{14}C-carbonyl-labeled carbaryl.

Carbaryl-^{14}C and chlordane-^{14}C residues appeared rapidly in the blood with maximum concentrations, 10 and 4% of dose, occurring after only 2 to 5 minutes. Then, the residues immediately began to dissipate from the blood. Leptophos and parathion treatments resulted in a somewhat slower accumulation of ^{14}C-residues in the blood but the maximum concentrations were similar to that observed with carbaryl. The organophosphorus residues did not dissipate after reaching peak concentrations as did those of carbaryl and chlordane. Rapid ester hydrolysis of carbaryl, leptophos and parathion was evidenced by the different levels of radiocarbon in the blood following treatment with the same chemical, but with the radioactive carbon on the acid or alcohol moieties. In each case, the levels of residues in the blood were almost doubled when the radiolabel was on the alcohol portion of the molecule.

Levels of ^{14}C-residues in selected tissues of the rats one hour after treatment are given in Table 18. Concentrations in the lungs were highest with leptophos and chlordane, 30 and 24% of the inhaled dose, while carbaryl and parathion residues were only one-third these levels. Results obtained with those compounds radiolabeled on the acid and alcohol moieties were very similar. Indicating that the ester linkages were intact.

With carbaryl, parathion, and leptophos, residues in the kidney and bladder at the end of 60 minutes were higher for the ^{14}C-alcohol-labeled insecticide than for the corresponding ^{14}C-acid-labeled compound. The liver, on the other hand, contained higher residues with the ^{14}C-acid-labeled materials, demonstrating that the acid moieties released upon hydrolysis were not removed from the liver as rapidly as the alcohol moieties. That carbaryl showed higher ^{14}C-equivalents in the liver suggests that there was a delay between the formation of methyl carbamic acid and its further degradation to carbon dioxide. Chlordane-^{14}C residues in the liver were equivalent to 20% of the dose but only trace levels were detected in the kidney or bladder (Table 18).

Table 18. RESIDUES IN TISSUES OF RATS 1 HOUR AFTER INHALATION OF ^{14}C-INSECTICIDES.

	% of administered radiocarbon/tissue			
Insecticide	Liver	Lung	Kidney	Bladder[a]
Carbaryl-ring-^{14}C	4.8	10.5	3.7	5.9
Carbaryl-carbonyl-^{14}C	9.2	9.2	2.9	2.7
Leptophos-phenoxy-^{14}C	8.9	31.8	3.9	4.8
Leptophos-phenyl-^{14}C	15.3	29.4	3.3	2.3
Parathion-ring-^{14}C	4.0	8.1	10.7	18.0
Parathion-ethyl-^{14}C	8.5	7.3	2.5	10.8
Chlordane	19.6	23.9	0.3	0.1

[a] Includes urine contained therein.

While there was rapid absorption of the inhaled compounds into the body following exhalation, the excretion rates also were quite rapid. Carbaryl, leptophos, and parathion equivalents were eliminated in the urine in amounts greater than 90% of the inhaled doses by 3 days. The feces contained an additional 2 5o 5% of the doses. For chlordane, excretion was primarily via the feces and was slower than the other compounds. About 52% of the dose was eliminated in the feces after 6 days, while the urine contained an additional 12%. The rates of excretion following inhalation are in good agreement with those reported for orally administered carbaryl (Knaak *et al.* 1965), chlordane (Barnett and Dorough 1974), parathion (Nakatsugawa *et al.* 1969), and leptophos (Holmstead *et al.* 1973). It would appear, therefore, that the ultimate fate of these insecticides when inhaled is not appreciably different than when the compounds enter the body by ingestion.

INSECTICIDE RESIDUES IN CIGARETTE SMOKE: TRANSFER AND FATE IN RATS

A degree of human exposure to insecticides and other types of pesticidal chemical residues is unavoidable because of their ubiquitous nature in the environment and particularly on food commodities. This source of contamination has long been recognized and stringent use limitations are established for each pesticide in an effort to assure that levels of exposure are kept well below those believed to be hazardous. Tobacco smoke is another possible source of human exposure to insecticides but their actual significance to the health of the smoker is largely unknown (Wynder and Hoffman, 1967). However, it is known that various insecticide residues occur in commercial tobacco products (Dorough and Gibson, 1972) and that insecticides and their pyrolysis products may be transferred to the mainstream smoke of cigarettes (Tso, 1972).

Even so, tobacco smoke has not been generally considered as an important source of human exposure to insecticides and, not being a food crop, tobacco is not included among those crops requiring official pesticide tolerances. Nevertheless, regulatory officials do carefully consider the levels and nature of residues in tobacco and in smoke condensates as part of the evaluation of pesticides whose proposed use includes the control of tobacco pests. If hazardous conditions appear likely, the use may be denied or proper limitations imposed to circumvent the potential problems involved. This type of consideration is relatively new and there is a critical need for information which will assist in determining the significance of pesticide residues in tobacco and tobacco smoke. A most obvious void in this regard is information relating to the fate of insecticide residues, their metabolites and/or pyrolysis products after being inhaled. Studies of this nature should be conducted with animals and, as is the case with pesticide residues on food crops, the data extrapolated to humans.

In the current study, a simple but quantitative smoking device was constructed whereby smoke containing radioactive insecticide residues was transferred directly from the cigarette to the lungs of rats via the trachea. Rats were selected because they are commonly used in metabolic fate studies of pesticides, and the existing information would be useful in interpreting the significance of data obtained in the smoke experiments. While it is desirable to administer the smoke to the animal in a more natural manner, an effective quantitative system that would allow the "smoking" of animals similar to the process in humans has yet to be developed. Enclosing animals in cylinders or chambers (Moore and Bock, 1956; Holland et al., 1958) may be sufficient for studying the pathological effects of chronic smoke exposure, but the quantitative aspects necessary for pesticide fate studies and for determining exposure potential of pesticides to the smoker are not provided by such methods. Forced smoking via the trachea (Wynder and Hoffman, 1967; Armitage et al., 1969) allows an excellent means of administering quantitative doses of smoke containing insecticide residues to the lungs of animals, and the subsequent determination of the fate of the inhaled residues.

Materials and Methods

Treatment of Cigarettes - Radioactive insecticides were used in all experiments. The identity of the compounds and the position of the ^{14}C-label on the molecule are shown in Table 19. These insecticides were selected to represent different chemical types and not as examples of compounds used for the control of tobacco pests. In fact, of those insecticides listed in Table 19, only carbaryl and carbofuran are currently approved for this use. DDT was registered for use on tobacco until 1970 and residues do exist in commercial cigarettes (Dorough and Gibson, 1972).

Reference research cigarettes 1R1 were provided by the Tobacco and Health Research Institute (University of Kentucky, Lexington, Kentucky). The desired quantity of insecticide was dissolved in 0.1 ml of acetone and applied to a 17 mm segment, extending from a point 25 mm from the

Table 19. RADIOACTIVE COMPOUNDS USED IN THE STUDY OF THE TRANSFER OF INSECTICIDE RESIDUES TO RATS IN CIGARETTE SMOKE.

Chemical identity and position of carbon-14	Designation	Specific Activity mC/mM
1-Naphthol-1-^{14}C	1-Naphthol-^{14}C	15.2
1-Naphthyl-1-^{14}C methylcarbamate	Carbaryl-naphthyl-^{14}C	0.6
1-Naphthyl-methylcarbamate (carbonyl-^{14}C)	Carbaryl-carbonyl-^{14}C	26.4
1-Naphthyl methyl-^{14}C carbamate	Carbaryl-methyl-^{14}C	1.1
2,3,-dihydro-2,2-dimethyl-7-benzo-^{14}C furanyl-N-methylcarbamate	Carbofuran-ring-^{14}C	2.85
2,3,-dihydro-2,2-dimethyl-7-benzofuranyl-N-methylcarbamate (carbonyl-^{14}C)	Carbofuran-carbonyl-^{14}C	3.83
O-(2,5-dichloro-4-bromophenyl-^{14}C) O-methyl phenylthiophosphonate	Leptophos-phenoxy-^{14}C	7.34
O-(2,5-dichloro-4-bromophenyl) O-methyl phenyl-^{14}C-thiophosphonate	Leptophos-phenyl-^{14}C	6.22
O,O-Diethyl-O-p-nitrophenyl-^{14}C phosphorothioate	Parathion	1.8
1,3,4-methenododecachlorooctahydro-2H-cyclobuta [c,d] pentalene (^{14}C UL)	Mirex-^{14}C	6.34
1,1,1-Trichloro-2,2-bis (p-chlorophenyl-^{14}C) ethane	DDT-^{14}C	2.7

butt end, of the cigarette using a micro syringe. The needle of the syringe was inserted into the cigarette 10 mm from the treatment zone towards the end to be lighted, passed through the center of the cigarette until the point was at the center of the 17-mm band, and the solution slowly injected into the cigarette. Each insecticide was applied to the 200 mg of tobacco in the treated zone at a concentration of 100 ppm. To evaporate the solvent, the cigarettes were left at room temperature for 2 hr and then refrigerated overnight before use. No degradation of the insecticides occurred during storage of the cigarettes. Impregnation of hundreds of cigarettes in this manner, and subsequent radioassay of subsections of the impregnated zone, showed that the radioactivity was distributed in a bell shape fashion with just trace amounts on either side of the marked zone.

Smoking Device - A smoking device was designed to collect, or transfer to the lungs of rats, known volumes of smoke at designated intervals. The mainstream smoke outlet was attached to a valve, (3-way Teflon valve, Hamilton ML-3000) which allowed a puff to be drawn into a syringe and then transferred to the lungs through a polyethylene tube, (1.6 mm ID, 2.1 mm OD). Manual operation of the 3-way valve and delivery of a 5-ml puff to the animals required 2 to 3 sec. A 50-ml syringe was attached to the tracheal tube by a 27G needle for the collection of the exhaled smoke. For mainstream smoke analysis, the smoke was collected directly in an air tight syringe at the end of the tracheal tube. For each test, a cigarette was placed in the holder, lit and the cover of the smoking chamber was put into place. Air was passed through the chamber at 500ml/min to remove the sidestream smoke and to keep the cigarette burning. Sidestream smoke was passed through traps of glass wool, cold ethanol, and 2 carbon dioxide trap solutions consisting of a 2:1 mixture of 2-methoxyethanol and 2-aminoethanol. After the appropriate smoking sequence, the air flow through the smoking chamber was stopped to extinguish the cigarette and then operated again momentarily to clear the chamber of any smoke before it was opened to collect the ash and butt.

Inhalation Studies - Female white rats (Sprague-Dawley) weighing 180-200 g and 9 to 10 weeks old were used in the smoking studies. For each inhalation experiment, an impregnated cigarette was placed in the holder, lit, and the smoking chamber cover put into place. Immediately, a rat was anesthetized with ether, and a midline longitudinal incision made to expose the trachea. A thread was introduced around the trachea and a small hole was clipped between the 4th and 5th tracheal rings caudal to the thyroid cartilage. Just as the cigarette burned to the treatment zone, the tracheal tube of the smoking system was introduced into the trachea and the thread was tied over the trachea and the tube. A 5-ml puff was drawn into syringe C and directed into the lung of the rat. The smoke was held in the lungs for 12 sec, then 5 ml were withdrawl into syringe E. The complete process required 15 sec. After repeating the process for a total of 8 puffs, during which the entire treatment zone was burned, the trachea was immediately clamped and the air flow through the system was stopped to extinguish the cigarette.

A blood sample was removed from the right ventricle of the heart and the lung and heart were removed and placed in a freezer. Blood was taken about 2.5 min after the last exhalation and the heart and lungs were excised about 1 min later.

Analysis - The blood was immediately radioassayed by combustion in a Beckman Biological Materials Oxidizer and the radioactive carbon dioxide trapped in a 2 to 1 mixture of 2-methoxyethanol and 2-aminoethanol. Total blood volume of the animals was calculated to be 12.5 ml. Heart and lung tissues were similarly analyzed after 1 hr.

Radioactive residues in the exhaled air were removed by washing the syringe with ethanol and carbon dioxide trap solution. First, the ethanol was pulled into the syringe, the syringe shaken thoroughly, and the ethanol forced from the syringe until only the needle was filled with solvent. An air space was left between the syringe pumper and solvent to contain any gases and/or other volatile components not soluble in ethanol. The ethanol wash was repeated, followed by 2 washings of the syringe with the carbon dioxide trap solution. This same procedure was used in collecting the mainstream smoke radiocarbon for direct analysis. The ethanol wash was concentrated and applied to thin layer plates (Merck, silica gel F-254). Solvent system for the different compounds were: carbaryl-7:3 benzene:ether; carbofuran-5:1 ether:hexane; letpophos-6:4 chloroform:benzene; mirex-n-heptane; DDT-n-hexane. Individual components resolved by tlc were quantitated but no attempt was made to identify the pyrolysis products. The radiocarbon suspected of being the original insecticide added to the cigarettes was extracted from the gel and rechromatographed as a mixture with the authentic compound. Cochromatography was achieved with each of the insecticides evaluated.

To determine if the radiocarbon in the carbon dioxide trap solution was actually ^{14}C-carbon dioxide, a barium hydroxide trap was used in place of the 2-aminoethanol and 2-methoxyethanol. Approximately the same quantity of material was trapped in the barium hydroxide solution and, upon acidification, about 90% of the radiocarbon dissipated. These data indicated that the radiocarbon was predominately ^{14}C-carbon dioxide and not other volatile components of the insecticides formed during the smoking process.

Radiocarbon in the ash, butt and smoking chamber was determined by washing or extracting the residues with ethanol. Aliquots of the liquid sidestream smoke traps were radioassayed directly while the glass wool was washed with ethanol and then the wash assayed. Ethanol solubles from the sidestream smoke, which included the smoking chamber washes, were analyzed by tlc as described for the mainstream smoke.

All radioassays were performed using liquid scintillation counting (Packard Tri-Carb Model 3380) and a commercial blend of scintillation counting fluid (3a70B, Research Products International Corp., Elk Grove Village, Ill.). All data reported herein were derived from a minimum of 5 replicates for each experimental parameter investigated.

Inhalation Parameters - During the development of the smoking process described above, various studies were performed to determine the effects of puff volume and lung retention time on the amount of ^{14}C-residues retained in the animal body. The objective was to obtain the maximum level of radiocarbon in the body following the smoking process so that its fate in the rats could be determined.

Cigarettes were impregnated with carbaryl-naphthyl-^{14}C as previously described and then the entire treated zone was smoked using 2.5-ml and 5-ml puff volumes. For each puff volume, the smoke was held in the lungs for 6, 9 and 12 sec. The number of puffs required to smoke the treated portion of the cigarettes decreased as the lung retention time was increased, with the numbers being 22, 18 and 16, respectively, when the 2.5-ml puffs were used. For the 5-ml puffs, the number required was reduced by one-half. Radiocarbon remaining in the body after the last smoke exhalation was determined by radioassay of the lungs, blood and heart.

In another experiment, the smoking device was modified by replacing the 3-way valve with an in-line flutter valve which opened as the animal inhaled, thus drawing smoke into the lungs, and closed when the animal exhaled, forcing the expired smoke into the collection syringe. This was a totally automatic process since the plunger of the syringe lubricated with ethanol, was pushed outward by exhalation but maintained its position during inhalation. Using this system, anesthetized animals smoked the treated zone of the cigarettes in approximately 180 puffs. The average single puff volume was 0.8 ml with the inhalations per min being between 85 and 95. This situation represented the minimum puff volume and lung retention time attainable with the smoking device used in these studies. Rats were allowed to smoke cigarettes impregnated with carbaryl-naphthyl-^{14}C by the self-inhalation method and the level of ^{14}C-residues remaining in the body were compared with those obtained with the standard eight 5-ml puff smoking process, and with a single inhalation of 5 ml of smoke.

The levels of carbaryl-naphthyl-^{14}C equivalents in the blood of animals during the self-inhalation and eight 5-ml-puff smoking process, and for 4 min after the last puff, were determined. Blood was drawn from the subclavian vein at various intervals after the first inhalation and the total radiocarbon in the samples determined by combustion analyses. In this particular study, 10 animals were used for each method of smoke exposure since considerable variation in the blood levels was encountered.

Initially, flutter valves were used in the smoking apparatus to administer smoke to the lungs of rats rather than using the manually operated 3-way valve. A flutter valve was inserted which allowed the smoke to be drawn into the syringe and then transferred to the lungs. Although this arrangement was superior to the 3-way valve in regard to ease of operation and rapidity of smoke delivery, it was discarded because 50 to 75% of the ^{14}C-residues in the mainstream smoke of 5-ml puffs was deposited directly on the valves. With the 3-way valve, which operates in a completely open or completely closed manner, the amount of mainstream smoke radiocarbon on the valve was generally less than 1%.

35-ml Puff Volumes - The use of rats in smoking studies for the purpose of estimating the fate of inhaled insecticide residues in humans would be valid only if the nature of the inhaled residues were the same, and if the amounts were comparable based on lung capacity. These factors were evaluated by determining the quantity and nature of ^{14}C-residues in the mainstream smoke of cigarettes impregnated with each of the insecticides, but not all radioactive preparations, listed in Table 19, and smoked using 35-ml puffs of 2 sec duration at 1 min intervals. This is the procedure normally used with commercial smoking machines to represent human smoking (Wynder and Hoffmann, 1967). The impregnated zones of the cigarettes were burned completely by 3 puffs. All other aspects of these studies (treatment of cigarettes, collection of smoke, analyses, etc.) were the same as reported for the 5-ml puffs.

Filters - The effect of filters on the quantity of carbaryl-naphthyl-^{14}C equivalents transferred to the mainstream smoke of cigarettes was evaluated using the eight 5-ml puff sequence. Four commercial brands of filter cigarettes were impregnated with the insecticide and the radiocarbon in the ash, butt, sidestream smoke and mainstream smoke was quantitated. Ethanol-soluble ^{14}C-residues in the mainstream smoke were analyzed by tlc. The same procedure was followed using cigarettes from the same package which had the filters removed. Reference cigarettes, 1R1, were included in the study with the butt length adjusted to be equivalent to the tobacco plus filter of the filter cigarettes. When the filters were removed, an equivalent length of the 1R1 cigarettes was also removed. Therefore, it was possible to compare the trapping effect of the filters with a comparable length of plain tobacco.

Commercial cigarettes used in these experiments, designated as brands A-D, each had a filter length of 20 mm except brand B which was 25 mm. The nature of the filters was as follows: Brand A, 2 cellulose filters separated by charcoal granules; Brand B, an inner cellulose filter (towards the burning end) containing charcoal granules and an outer cellulose filter; Brand C, an inner charcoal-impregnated cellulose filter and an outer cellulose filter; Brand D, a single cellulose filter. The treatment zone of the filter cigarettes, 17 mm, was 25 mm from the point where the filter was joined to the cigarettes, making the tobacco-containing butt the same length as for non-filter cigarettes.

Fate of Inhaled Residues - In one series of experiments, the tracheal tube was disconnected from the smoking apparatus immediately after administering the smoke to the rats and attached to a Y connector, one end of which led to a carbon dioxide trap while the other end was open to the atmosphere to provide air to the animal. The carbon dioxide trap solution, 2:1 methylcellosolve-ethanolamine, was radioassayed at 5 min intervals for the first 15 min, then at 15 min intervals until the experiment was terminated. Meanwhile, 0.25 ml blood samples were collected from the subclavian vein at approximately 3 min intervals for the first 10 min, then at 10 min intervals thereafter. At the end of the experiment, 90 min, the urinary bladder was ligated and removed intact. The other organs and tissues were immediately excised and frozen.

In another series of experiments, the tracheal tube was quickly withdrawn after the last of eight 5-ml smoke inhalations and exhalations. The incision in the skin was then closed with a wound clip and the rat immediately placed in a metabolism chamber (Nuclear Associates, Inc., N. Y.). Inhalation-quality compressed air was passed through the chamber at the rate of 200 ml/min and then through a carbon dioxide trap solution. The feces and urine were collected separately. At the end of 6 hr, the animal was transferred to a larger metabolism cage for 4 days and then sacrificed. Various tissue samples were taken and immediately frozen.

The urine samples were monitored for total radiocarbon by direct scintillation counting of 0.5 ml in 10 ml of a commercial blend of scintillation counting fluid (3a70B, Research Products International Corp., Elk Grove Village, Ill.). Urine samples having 500 dpm/ml or more were pooled and lyophilized to approximately 1 ml, then applied to thin layer plates (Merck, silica gel F-254). Radioactive residues in the feces were quantitated by combustion followed by scintillation counting of the carbon dioxide trap solution.

The deposition of the insecticide residues in the rat body were investigated after 90 min and 4 days. Each sample was weighed and combusted in a Beckman Biological Materials Oxidizer. For calculation purposes, a 200 gm female rat was also dissected and different tissues and organs separated and weighed. The percent of total body weight for the tissues was as follows: lung, 1.2; heart, 0.5; skin, 14.2; hair, 2.1; muscles, 39.8; fat, 9.1; digestive system, 7.8 (3.4 without food); liver, 3.7; urinary system, 1.0; reproductive system, 0.5; spleen, 0.2; brain, 0.9; skeleton, 11.9 (bones, 7.9, and cartilage, 4.0); tongue, 0.4; total weight 99.6%. The total radiocarbon in the body was based on these actual weights and on previous reports in the literature.

Pyrolyses of Carbofuran - Controlled pyrolyses were conducted in an apparatus recently designed by Smith and Patterson (1973) to more closely correlate with the burning process in cigarettes. A 9 mm x 170 cm horizontal quartz tube in which the pesticide had been evenly distributed over an 80-cm segment, was connected to 2 cold traps, dry ice-chloroform, and swept with nitrogen at 40 ml/min. The oven consisted of a nickel rod, 44 mm in diameter and 50 mm in length, supported in an insulated aluminum cabinet (Chemical Data Systems, Oxford, Penn.). The nickel rod was drilled to provide for three 150 watt 1/4" x 2" Chromalox CIR cartridge heaters (Emerson Electric Company, Pittsburg, Penn) and an integrated platinum temperature sensor-thermocouple cartridge. A 13 mm hole was drilled through the center of the rod to accomodate the combustion tube. Temperatures of 650° and 700°C were maintained by a CDS-200 power-proportioning temperature controller (Chemical Data Systems). The oven, attached to a moveable plate, was driven at 2.7 cm/sec along the quartz tube containing the insecticide.

A vertically positioned Vycor combustion tube (2.5 cm x 94 cm) containing 20 cm of Berl Saddles positioned in the heated zone was used to represent the more conventional pyrolysis conditions. The tube was connected to cold traps and the system swept with nitrogen. The combustion tube was heated to 783°C by a Lindberg furnace.

Noncondensable gases were collected in a 10 cm IR gas cell (Wilks Scientific, South Norwalk, Conn.) and the spectra recorded on a Beckman IR 8. Precoated silica gel plates were used for tlc analyses of the components of the cold traps. After development in 7:3 benzene-methanol, the spots were visualized under ultraviolet light or by spraying with 0.5% methanolic p-nitrobenzenediazonium fluoroborate. GLC analyses were performed on a Varian 1400 instrument equipped with an FID. Neutral pyrolysis products were examined on 4 mm x 180 cm glass column packed with 5% OV-101 while the acidic ones were analyzed on a column packed with 10% EGSSX on Gas Chrom Q. Injector and detector were maintained at 210 and 22°C. Column temperatures were programmed from 100° to 230°C at 1°/min with the OV-101 column and 128°C to 200°C at 4°/min for the EGSSX support. The nitrogen carrier gas flow was 35 ml/min. GC/MS data were obtained on the Finnigan 1015C at 70 eV, and the NMR data on a Varian HA-60 spectrometer interfaced with a Varian 1024 computer.

Results and Discussion

Inhalation Parameters - Retention of carbaryl-naphthyl-^{14}C equivalents in the body of rats immediately after exposing the animal to cigarette smoke using different puff volumes, numbers, and lung-contact times is shown in Table 20. Of primary importance in this regard was the period of time the smoke was held in the lungs. Maximum retention of the radiocarbon, 76% of that inhaled, was obtained with a single 5-ml puff held in the lungs for 12 sex. Of course, the actual amount of carbaryl-^{14}C equivalents in the body after the single puff was very small, only 0.9% of that radioactivity added to the cigarette. Repeating the 5-ml puff 8 times in the same manner, during which the entire impregnated zone of the cigarette was smoked, increased the total carbaryl-^{14}C equivalents inhaled by a factor of 9 and the amount retained by a factor of 8. This smoking sequence resulted in 7.5% of the radioactivity added to the cigarette being retained in the lungs, blood and heart. Unlike the single 5-ml puff smoking process where 78% of the retained residues were in the lungs, the eight 5-ml puffs resulted in almost equal amounts

Table 20. EFFECT OF SMOKE EXPOSURE METHOD ON RETENTION OF CARBARYL-NAPHTHYL-^{14}C EQUIVALENTS IN THE BODY OF RATS IMMEDIATELY AFTER THE SMOKING PROCESS.

Puffs[a]			% of ^{14}C in smoked portion of cigarette					
				Retained in body[b]				
Number	ml	Sec held in lung/puff	Exhaled	Lung	Blood	Heart	Total	Total
1	5.0	12	0.3	0.7	0.2	0.03	0.9	1.2
180[c]	0.8	0.7	4.1	0.2	1.9	0.05	2.2	6.3
22	2.5	6	5.6	1.0	2.4	0.21	3.6	9.2
18	2.5	9	4.1	1.6	2.6	0.33	4.5	8.6
16	2.5	12	2.8	2.1	3.1	0.37	5.6	8.4
11	5.0	6	4.6	2.0	4.5	0.32	6.8	11.4
9	5.0	9	3.7	2.1	4.6	0.44	7.1	10.8
8	5.0	12	3.3	3.5	3.7	0.30	7.5	10.8

[a] With the exception of the single 5-ml puff, the number of puffs represents that necessary for the animal to smoke the entire impregnated zone, 17 mm, of the cigarette.

[b] Trachea clamped after exhalation of last puff. Blood collected 2.5 min later; lung and heart excised after 3.5 min.

[c] Rats permitted to smoke at normal respiration rate of anesthetized animals.

of residues in the lungs and blood. Because the quantity of radiocarbon remaining in the body using eight 5-ml puffs was greater than with any other smoking procedure, the process was selected as the standard method of exposing rats to insecticide residues in cigarette smoke.

In addition to establishing conditions for maximum body retention of ^{14}C-insecticide residues inhaled in cigarette smoke, the data in Table 20 indicate some of the factors effecting the degree of transfer and retention of the residues. For example, while the percentage transfer of carbaryl-^{14}C equivalents to the mainstream smoke was obviously greater as the total volume of smoke increased, there was very little difference when considered on a per ml basis. This was true not only for carbaryl, but for each of the insecticides evaluated, and for each puff volume used (Table 21). Under conditions used in these experiments, each ml of mainstream smoke contained an equivalent of between 0.2 to 0.3% of the radioactive insecticide in the tobacco burned during the smoking process. Increasing the number of puffs required to burn the same amount of tobacco did tend to reduce the percentage transfer or residues to the mainstream smoke. However, this reduction was grossly evident only when the number of puffs was 180, that required to burn the impregnated zone using the normal respiration rate of anesthetized rats (Table 20).

Retention of the inhaled ^{14}C-residues was primarily a function of the period of time the smoke was held in the lungs, and to a lesser extent to the volume of the puff. Increasing either of these parameters resulted in increased retention of the inhaled radiocarbon. Of the retained residues, less was located in the blood as the lung contact time of each puff was increased from 6 to 12 sec; the percentage remaining in the lung tissue showed a corresponding increase. Therefore, these data show that, with repeated inhalation, the deposition of insecticide equivalents in the lungs exceeded the rate of their removal by the blood.

Table 21. DISTRIBUTION OF RADIOCARBON FOLLOWING SMOKING OF CIGARETTES WITH 5-ML AND 35-ML PUFFS. PERCENT ADDED TO CIGARETTE[a].

Compound and			Sidestream	Mainstream smoke					Total
					Traps				
puff volume	Ash	Butt	smoke	Apparatus	Ethanol	CO_2	Total	Total	recovery
1-Naphthol-^{14}C, 5-ml	0.3	19.8	60.1	1.3	7.9	1.6	9.5	10.8	91.0
1-Naphthol-^{14}C, 35-ml	0.2	23.1	48.3	6.0	13.3	1.5	14.8	20.8	92.4
Carbaryl-naphthyl-^{14}C, 5-ml	0.4	16.5	63.8	1.0	7.4	1.8	9.2	10.3	91.0
Carbaryl-naphthyl-^{14}C, 35-ml	0.2	20.5	51.1	5.1	20.1	2.0	22.1	27.2	99.0
Carbaryl-carbonyl-^{14}C, 5-ml	0.3	7.8	67.4	0.7	5.9	2.5	8.4	9.1	84.6
Carbaryl-methyl-^{14}C, 5-ml	0.5	14.8	71.2	0.8	7.5	0.8	8.3	9.1	95.6
Carbofuran-ring-^{14}C, 5-ml	0.5	13.8	68.8	0.8	7.1	1.4	8.5	9.3	92.4
Carbofuran-ring-^{14}C, 35-ml	0.4	18.8	53.2	4.0	22.4	1.5	23.9	27.9	100.3
Carbofuran-carbonyl-^{14}C, 5-ml	0.2	10.7	76.5	0.8	6.4	0.7	7.1	7.9	95.3
Leptophos-phenoxy-^{14}C, 5-ml	0.5	13.6	66.3	1.2	10.1	3.2	13.3	14.5	94.9
Leptophos-phenoxy-^{14}C, 35-ml	0.4	21.6	38.8	4.9	20.8	1.7	22.5	27.4	88.2
Leptophos-phenyl-^{14}C, 5-ml	0.2	7.3	52.6	0.7	4.4	2.0	6.4	7.1	67.2
Mirex-^{14}C, 5-ml	0.2	19.6	61.2	1.1	9.0	2.6	11.6	12.7	93.7
Mirex-^{14}C, 35-ml	0.4	25.0	40.6	4.2	23.9	3.9	27.8	32.0	98.0
DDT-^{14}C, 5-ml	0.5	20.2	65.4	1.1	8.7	2.0	10.7	11.8	97.9
DDT-^{14}C, 35-ml	0.4	22.6	50.0	4.4	21.7	2.4	24.1	28.5	101.5

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[a] 17 mm portion of cigarette impregnated with 100 ppm insecticide. Entire impregnated zone smoked using eight 5-ml puffs or three 35-ml puffs.

Following the smoke-exposure period, carbaryl-^{14}C equivalents continued to increase in the blood. Maximum concentrations were approximately 28% of the total radiocarbon inhaled with both the eight 5-ml puff smoking process and when the animals were exposed to the smoke at normal respiration rates. Blood of rats exposed to smoke by normal respiration rates of anesthetized animals contained maximum levels of radioactivity 1.5 min after the last smoke exhalation, and then the levels began to dissipate gradually. Peak concentrations of carbaryl-^{14}C equivalents in the blood of rats given the eight 5-ml puffs were reached after 3.5 min, and remained unchanged after 4 min, or 6 min after initiating the smoking process. In these experiments, the rats were allowed to continue breathing after smoke exposure, but for all other data reported herein, the trachea was clamped immediately after the last smoke exhalation. Blood samples were then collected after about 2.5 min. Levels in the blood when sampled in this manner were approximately twice that at the end of the smoking sequence. That the accumulation of carbaryl-^{14}C equivalents in the blood was not appreciably altered by clamping the trachea was demonstrated by the fact that ^{14}C-residue levels in the blood were very similar when the trachea was clamped (Table 20) and when the animals were permitted to continue breathing. This is in agreement with the observation that the heart functioned for about 3 min after clamping the trachea.

Smoking Process and Transfer to Mainstream Smoke - The impregnated zone of the cigarettes weighed 200 ± 29 mg while the amount of tobacco burned per 5 ml puff, including the interval between puffs, was 25 ± 3.8 mg and contained 12.5 ± 6.2% of the compound added to each cigarette. Variation in the amount of ^{14}C-insecticide equivalents per puff was caused largely by the bell-shaped distribution of the insecticide in the spiked zone. Such variations probably did not effect retention of the insecticide equivalents in the rat lung since Egle (1970) reported no difference in acetaldhyde retention in humans with up to 50% variation in concentration. A similar lack of concentration effect was noted in our studies using carbaryl at 25 and 100 ppm.

One of the major objectives of this study was to perfect a simple, reproducible system capable of comparing the potential of different insecticides to be transferred in a toxic form to humans in tobacco smoke. Toxic residues on the leaf at market or in smoke condensates are significant only if they survive the smoking process and are retained in the body at sufficient levels and periods to induce pathological conditions. Based upon the excellent accountability of the radiocarbon added to the cigarettes (Table 21), it appears that the procedure described herein could serve as a general method for estimating, at least in part, the significance of pesticides and their metabolites in tobacco products. With the exception of leptophos-phenyl-^{14}C where the recovery was only 67%, total recovery of the compounds ranged from 85% for carbaryl-carbonyl-^{14}C to 102% for DDT. The loss of radioactivity with certain of the insecticides indicated that volatile pyrolysis products were formed which were not collected in the traps of the smoking apparatus.

There was no consistent difference in the recovery of the insecticides using puff volumes of 5 and 35 ml (Table 21). Neither were there any great differences in the percentage transfer of pesticide-^{14}C equivalents to the mainstream smoke when considered on a per ml basis. With the 5-ml puffs, 40 ml total, from 7 to 14% transfer occurred, whereas with the 35 ml puffs, 105 ml total, the transfer to the mainstream smoke was 21 to 32%, resulting in an overall transfer of 0.18 to 0.34% per ml of smoke. Therefore, the quantities of residues inhaled by rats given 5-ml puffs would be comparable in regard to lung capacity to that inhaled in 35 ml puffs by man. With the 35-ml puffs, more of the ^{14}C-residues were deposited in the butt and less given off in the sidestream smoke than with the 5-ml puffs. Only minute residues were in the ash of the cigarettes, regardless of the puff volume or compound involved.

The use of carbaryl, carbofuran and leptophos radiolabeled at different positions on the molecule showed that the distribution of radiocarbon following smoking of the cigarettes varied little with the position of the radioactive carbon. Carbaryl-naphthyl-^{14}C and carbaryl-methyl-^{14}C behaved similarly to 1-naphthol-^{14}C, but with carbaryl-carbonyl-^{14}C, the levels of residues in the butt were much less. This apparently resulted from the production of ^{14}C-carbon dioxide from the carbonyl carbon which did not condense in the butt as did the other ^{14}C-labeled pyrolysis products. With the exception that radioactive residues in the sidestream smoke were higher with carbofuran-carbonyl-^{14}C, the distribution of residues was about the same as for carbofuran-ring-^{14}C. In the case of leptophos, the phenyl-labeled material gave lower residues in all fractions than did the phenoxy-^{14}C material. Obviously, some phenyl-^{14}C pyrolysis product(s) was generated which escaped the sidestream and/or mainstream smoke traps.

To compare the distribution and nature of residues in the mainstream smoke using our smoking apparatus with that of a commercial smoking machine, leptophos-phenoxy-^{14}C was added to cigarettes and smoked with both systems using 35-ml puffs. With the syringe method, a 17 mm portion of the cigarette was impregnated with 100 ppm leptophos in the manner already described. Cigarettes prepared for use on the commercial smoking machine were impregnated throughout their entire length at the same concentration. These latter cigarettes were smoked, 35-ml puffs of 2 sec duration at 1 min intervals, to a butt length of approximately 25 mm and the mainstream smoke, butts and ash were analyzed. In both cases, the distribution of radiocarbon was calculated on the basis of the leptophos-^{14}C content of the tobacco actually burned during smoking. Results of these analyses showed that the distribution of radiocarbon in the 3 fractions, and the nature of the ^{14}C-residues in the mainstream smoke, were virtually the same as obtained using our smoking apparatus (Table 21).

As mentioned earlier, 35-ml puffs are used to represent that of man, and have been used in previous studies of the transfer of insecticides to the mainstream smoke of cigarettes. One such study with DDT (Hoffmann and Rathkamp, 1968), showed that 12.4% of the parent compound was transferred to the mainstream smoke. Our 35-ml puff experiment gave almost identical results, showing 12.1% transfer of the applied material. Hengy and Thirion (1971) showed that the amount of the chlorinated hydrocarbon, endosulfan, transferred intact to the mainstream smoke was 15% of that contained in the burned tobacco. This is in contrast to the 3% transfer reported in another study (Guthrie and Bowery, 1962). Some of the difference in the 2 values may be explained by the fact that in the latter study, calculations were based on radioactivity added to the total cigarette and not restricted to that in the burned tobacco. Endosulfan was not evaluated in the present study, but with the chlorinated material mirex, 23% was as unaltered compound in the mainstream smoke.

In our studies, 10% of the organophosphorus insecticide leptophos was transferred intact to the mainstream smoke. With the carbamates carbaryl and carbofuran, the transfer was 11 and 20%, respectively. These values are far in excess of the 1% transfer reported for carbaryl and guthion, an organophosphate, in the review article by Guthrie (1968). However, they are similar to the 9% transfer of intact malathion, a rapidly biodegradable organophosphate, obtained in the studies by Hengy and Thirion (1970).

Mainstream Smoke - Smoking the ^{14}C-ring-insecticide-impregnated cigarettes with 35-ml puffs generally did not degrade the compounds as extensively as did the 5-ml puffs (Table 22). Only with carbaryl and DDT were the percentages of parent compound less with the higher puff volume. Mirex was the most stable compound with approximately 70% of the ^{14}C-components in the mainstream smoke as the applied compound. Three pyrolysis products were detected, the major one constituting 52% of their total. This product chromatographed similar to the monodechlorinated derivative of mirex formed by photolysis (Gibson et al., 1972). As with all other compounds evaluated, the number and relative magnitudes of the mirex pyrolysis products were similar with 5-ml and 35-ml puff volumes.

Table 22. NATURE OF RADIOCARBON IN MAINSTREAM SMOKE OF CIGARETTES IMPREGNATED WITH RING-^{14}C-INSECTICIDES.

	Percent of total ^{14}C-residues in mainstream smoke/puff volume[a]											
	1-Naphthol		Carbaryl		Carbofuran		Phenoxy-^{14}C Leptophos		Mirex		DDT	
^{14}C-Material	5-ml	35-ml	5-ml	35-ml	5-ml	35-ml	5-ml	35-ml	5-ml	35-ml	5-ml	35-ml
Unaltered compound	53.2	66.4	45.2	41.5	62.5	71.7	20.9	34.6	68.7	71.7	44.1	42.4
Carbon dioxide	14.8	7.2	17.5	7.4	15.0	5.4	22.1	6.2	20.5	12.2	16.9	8.4
Pyrolysis products[b]	32.0	23.0	31.9	44.8	10.8	8.7	50.7	54.2	4.0	7.7	32.1	38.9
Loss[c]	0	3.4	5.4	6.3	11.7	14.2	6.3	5.0	6.8	8.4	6.9	10.3

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[a] 17 mm portion of cigarette impregnated with 100 ppm insecticide. Entire impregnated zone smoked using eight 5-ml puffs or three 35-ml puffs.

[b] Number of pyrolysis products were 6, 7, 2, 4, 3, and 7 for the above compounds, respectively.

[c] Loss of ^{14}C Materials during tlc analysis.

Carbofuran was almost as stable as mirex, but only 2 pyrolysis products of carbofuran were detected by tlc analysis. The products existed in approximately equal concentrations and one chromatographed the same as the phenolic derivative of carbofuran.

The percentage of the ^{14}C-residues in the mainstream smoke as the applied compound, 40 to 45%, was the same with carbaryl-naphthyl-^{14}C and DDT. Both compounds yielded 7 pyrolysis products, with 66% of the pyrolysis products of carbaryl consisting of 1-naphthol. DDT has 2 major degradation products occurring in almost equal quantities which together constituted 64% of the pyrolytic materials resolved by tlc. 1-Naphthol was degraded during the smoking process to form all but 1 of the pyrolysis products observed with carbaryl-naphthyl-^{14}C. However, more 1-naphthol survived the smoking process than did intact carbaryl.

Unaltered leptophos-phenoxy-^{14}C accounted for less of the total ^{14}C-residues in the mainstream smoke than with any of the other compounds. Only 21% was as the parent compound with 5-ml puff volumes and 35% with 35-ml puffs. Four pyrolysis products detected by tlc analysis made up about 50% of the mainstream smoke ^{14}C-residues. Of these, 90% was as a compound with the same chromatographic behavior as the phenolic analog of leptophos.

Radioactive carbon dioxide accounted for 15 to 20% of the ^{14}C-residues in the mainstream smoke when cigarettes were smoked with 5-ml puffs (Table 22). With the 35-ml puffs, the amount of ^{14}C-carbon dioxide ranged from 5% to 12% of the total residues in the smoke. Since more complete degradation, especially to carbon dioxide, of the insecticides took place using eight 5-ml puffs than with three 35-ml puffs, it is apparent that exposure of the compounds to the 850-900°C heat at the burning point of the cigarette was greater with the smaller puff volume. Actually, this is probably a function of puff numbers, or frequency, and not to the volume of the puff. The amount of parent compound in the mainstream smoke clearly shows that much of the intact insecticides were volatilized away from the intense heat, and suggests that extensive pyrolysis occurred only to that material "trapped", or not volatilized, from the burning zone. This trapping effect apparently takes place to a greater degree during the initial phase of each puff, with increased volatility of the insecticides occurring as the temperature rises in the area just behind the burning zone of the cigarette. Therefore, less pyrolysis of the insecticide would be expected when the same amount of tobacco was burned with 3 puffs than with 8 puffs.

Sidestream Smoke - Insecticide residues in the sidestream smoke of cigarettes may serve as a source of contamination to the non-smoker as well as to the smoker. Analysis of the sidestream smoke of insecticide-^{14}C-impregnated cigarettes smoked with eight 5-ml puffs showed that the smoke contained considerable quantities of intact pesticide (Table 23). Intact leptophos constituted 21% of the sidestream smoke ^{14}C-residues, the same as it did in the mainstream smoke. In fact, the entire composition of leptophos-phenoxy-^{14}C equivalents in the sidestream smoke was almost the same as in the mainstream smoke. With all other compounds, the percentage of the residues in the sidestream smoke as the applied material was 2- to 4-fold less than that in the mainstream smoke; conversion of the insecticides to ^{14}C-carbon dioxide was much greater in the sidestream smoke.

Pyrolysis products of the insecticides, as determined by tlc analysis of the components of the glass wool and ethanol traps, were identical in number and similar in chromatographic behavior in both sidestream and mainstream smoke. No attempt was made to establish that the pyrolysis products were actually the same, but R_f values of corresponding products were similar enough to suggest this possibility. Ratios of the pyrolysis products of each insecticide were different in the sidestream smoke than in the mainstream smoke as evidenced by the autoradiographs; the products in the sidestream smoke were not individually

Table 23. NATURE OF RADIOCARBON IN SIDESTREAM SMOKE OF CIGARETTES IMPREGNATED WITH RING-^{14}C INSECTICIDES AND SMOKED USING EIGHT 5-ML PUFFS

	Percent of total ^{14}C-residues in sidestream smoke					
^{14}C-Material	1-Naphthol	Carbaryl	Carbofuran	Phenoxy-^{14}C Leptophos	Mirex	DDT
Unaltered compound	17.1	14.9	16.7	20.7	37.1	22.6
Carbon dioxide	62.1	61.4	45.9	28.5	36.4	35.5
Pyrolysis products	15.1	18.2	10.0	45.4	17.0	28.6
Loss	5.7	5.5	27.4	5.4	9.5	13.3

quantitated.

Loss of radioactivity during the analysis of the sidestream smoke was generally of the same magnitude as observed in the mainstream smoke analysis. The major exceptions were with carbofuran and DDT where greater loss occurred during analysis of the sidestream smoke.

Effect of Filters - Different types of commercial filters reduced the carbaryl-naphthyl-^{14}C equivalents in the mainstream smoke by 36 to 49% as compared to the same cigarettes without filters (Table 24). Increasing the butt length of unfiltered 1R1 cigarettes to that equivalent to the filter lengths reduced the mainstream smoke ^{14}C-residues by 28%. Therefore, the reduction of ^{14}C-residues as the result of filters was only 8 to 21% more effective than an equivalent length of tobacco. Two of the charcoal containing filters, A and C, were more efficient in trapping the residues than filter D which had no charcoal. However, the third charcoal-containing filter, B, was slightly less efficient than filter D. Very little selectivity was demonstrated by any of the filters. The reduction in the amount of radiocarbon in the mainstream smoke resulted from partial trapping of the parent compound and of the pyrolysis products except ^{14}C-carbon dioxide. Consequently, the ^{14}C-carbon dioxide accounted for a greater percentage of the mainstream smoke radiocarbon with the filter cigarettes than with the non-filtered ones.

Exhalation of Residues - During the smoking period, 26.2 to 41.9% of the total inhaled radiocarbon contained in the mainstream smoke was exhaled (Table 25). Of the individual ^{14}C-residues, 20.7 to 46.7% of the unaltered insecticides and 38 to 45% of gaseous pyrolysis products (of which at least 85% was $^{14}CO_2$) were expired. The extent of exhalation of nongaseous ^{14}C-pyrolytic products during smoking (about 20%) was similar for carbaryl, leptophos, parathion and DDT. Non gaseous residues of carbofuran exhibited nearly twice the clearance rate. Most of the exhalation of the residues occurred within the first 12 min

Table 24 . EFFECT OF FILTERS ON THE TRANSFER AND NATURE OF CARBARYL-NAPHTHYL-^{14}C EQUIVALENTS IN THE MAINSTREAM SMOKE OF CIGARETTES SMOKED WITH EIGHT 5-ML PUFFS.[a]

		^{14}C-Components of mainstream smoke: $\frac{\%\text{ of total }^{14}\text{C with filters}}{\%\text{ of total }^{14}\text{C without filters}}$		
Brand	% Reduction[b]	Carbaryl	CO_2	Pyrolysis products
A	48.6	.7	1.4	.9
B	35.8	.9	1.2	.9
C	42.5	.9	1.4	.8
D	37.8	.7	1.6	1.0
1R1[c]	28.4	.9	1.3	.9

[a] Cigarettes from same package smoked with and without filters.

[b] Reduction in the percentage transfer of radiocarbon from the burned tobacco to the mainstream smoke of filter cigarettes as compared with the same cigarettes with filter removed.

[c] Reference cigarettes, unfiltered, with butt length the same as other cigarettes with and without filters.

Table 25. RADIOACTIVE RESIDUES IN THE INHALED AND EXHALED SMOKE OF CIGARETTES FORTIFIED WITH ^{14}C-RING LABELED INSECTICIDES[a].

		Percent of radiocarbon in burned portion of cigarette[b] appearing in mainstream smoke as:				
Insecticide	Smoke	Parent Compound	Nongaseous Pyrolysis Products	Gaseous Pyrolysis Products[d]	Loss	Total ^{14}C-residues
Carbaryl	Inhaled	4.6	3.3	1.8	0.6	10.3
	Exhaled	1.1 (26.0)	0.6 (18.1)	0.7 (38.9)	0.3	2.7 (26.2)
Carbofuran	Inhaled	5.8	1.0	1.4	1.1	9.3
	Exhaled	2.3 (39.6)	0.4 (40.0)	0.6 (42.8)	0.6	3.9 (41.9)
Parathion	Inhaled	5.3	1.7	1.1	1.0	9.1
	Exhaled	1.1 (20.7)	0.3 (17.6)	0.5 (45.4)	0.3	2.2 (24.2)
Leptophos	Inhaled	3.0	7.4	3.2	0.9	14.5
	Exhaled	1.4 (46.7)	1.6 (21.6)	1.3 (40.6)	0.1	4.4 (30.3)
DDT	Inhaled	5.2	3.8	2.0	0.8	11.8
	Exhaled	1.7 (32.3)	1.0 (26.3)	0.8 (40.0)	0.2	3.7 (31.3)

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[a] Exhaled smoke collected during the smoking period using eight 5-ml puffs at 15 sec intervals.

[b] A 17 mm zone fortified with 200 ppm ^{14}C-insecticide. Values in parentheses are percent exhaled.

after smoking, but traces of radiocarbon were detected in the exhaled air for up to 5 hr (Table 26). Elimination of ^{14}C-residues through exhalation removed 64.7% of the inhaled carbofuran products and about 50% of the leptophos, carbaryl, and DDT residues. This route of elimination of parathion residues was less, with only 35% being removed.

Residues in the Respiratory and Circulatory Systems - Immediately following smoking, 22.8 to 39.5% of the inhaled radiocarbon was in the lungs (Table 26). After 1.5 hr, more than 90% of the residues had been removed by further exhalation and by absorption into the blood. Four days later, only 0.6% of the radioactivity in the inhaled mainstream smoke from DDT fortified cigarettes was detected, while lesser amounts were observed from the inhalation of carbaryl and parathion residues. Residues of leptophos and carbofuran were not detected.

Inhaled ^{14}C-residues accumulated rapidly in the blood during the smoking period and for 3 min thereafter. Peak levels of 39.8%, 36.0% and 27.3% of total inhaled radiocarbon from leptophos, parathion, and DDT residues respectively were observed. ^{14}C-Carbofuran and carbaryl pyrolysis products reached maximum blood concentrations of 18.1 and 32.5% of the inhaled residues during this time. Levels of ^{14}C-carbamate residues, carbaryl and carbofuran, corresponding to 28 to 30% of peak levels were detected in the blood after 90 min. Blood levels of leptophos, parathion, and DDT ^{14}C-residues exhibited a more rapid decline with only 9 to 12% of the maximum values being observed after 90 min. After 4 days, parathion and leptophos residues were not detected in the blood, whereas 0.2 to 0.3% of the inhaled radiocarbon from carbaryl, carbofuran, and DDT fortified cigarettes still remained.

^{14}C-Residues in the heart immediately following smoking ranged from 0.4 to 3.6% of the inhaled activity in the mainstream smoke. After 90 min, the amount had declined by approximately 75 to 85%. No residues were detected in the heart after 4 days.

Table 26. FATE OF ^{14}C-RING LABELED INSECTICIDE RESIDUES INHALED BY RATS IN CIGARETTE SMOKE.

Substrate and time after exposure	Percent of total radiocarbon inhaled/compound[a]				
	Carbaryl	Carbofuran	Parathion	Leptophos	DDT
Exhaled air, cumulative					
2 minutes[b]	26.2	41.9	24.2	30.3	31.3
12 minutes	41.5	52.3	28.6	35.3	40.1
90 minutes	47.3	64.0	34.6	48.0	50.8
5 hours	47.8	64.7	35.3	48.7	50.8
Urine, cumulative					
90 minutes	11.8	8.1	39.5	18.6	0.2
24 hours	42.4	21.6	55.0	28.3	1.9
4 days	48.4	40.1	58.8	32.2	4.0
Feces, cumulative					
4 days	2.4	1.5	1.6	9.8	7.4
Lung					
3 minutes	31.4	22.8	29.8	31.8	39.5
90 minutes	1.4	0.9	1.2	1.6	2.7
4 days	0.2	0.0	0.1	0.0	0.6
Blood					
3 minutes	27.2	17.8	36.0	39.8	27.1
90 minutes	9.6	5.0	4.5	3.8	2.9
4 days	0.2	0.2	0.0	0.0	0.3
Heart					
3 minutes	2.4	3.6	0.4	3.6	2.1
90 minutes	0.2	0.1	0.1	0.3	0.7
4 days	0.0	0.0	0.0	0.0	0.0
Liver					
90 minutes	5.5	3.3	2.6	3.2	6.7
4 days	0.6	0.3	0.0	0.0	2.5
Kidney					
90 minutes	1.8	2.8	2.0	2.5	0.7
4 days	0.1	0.0	0.0	0.2	0.1
Muscle					
90 minutes	3.5	6.3	1.3	1.6	20.0
4 days	0.0	0.0	0.0	0.0	11.4

Table 26 . continued

	Carbaryl	Carbofuran	Parathion	Leptophos	DDT
Fat					
90 minutes	9.1	3.8	10.4	1.5	9.7
4 days	0.5	0.0	0.9	0.8	33.1
Other tissues[c]					
90 minutes	0.4	7.3	0.6	0.3	1.2
4 days	0.0	0.0	0.0	0.0	0.7
Total recovery					
90 minutes	90.6	101.6	96.8	81.4	95.6
4 days	100.2	106.8	96.7	91.7	110.9

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[a] Each value represents the average obtained from 4 animals.

[b] Collected during smoking period.

[c] Includes spleen, brain, ovaries and uterus.

Residues in Urine and Feces - Urinary excretion removed 4.0 to 58.5% of the total inhaled ^{14}C-insecticide residues (Table 26). With carbaryl, carbofuran, and parathion, this amounted to 93 to 98% of the residues retained in the rat after the smoking process. In the case of leptophos, 78% of the retained ^{14}C-material was removed in the urine, while 23% was found in the feces. Clearance of DDT residues from the rat was much slower with only 7 and 12% of the residues excreted in the urine and feces after 4 days.

Tlc analysis of the urine revealed that 93% and 84% of the excreted carbaryl and carbofuran residues were polar materials. The apolar carbaryl metabolites, 7%, contained 4 products, none of which was carbaryl. Four apolar metabolites and the intact insecticide composed 16% of the carbofuran residues excreted. The parent compound accounted for 6.2% while the other residues, tentatively identified as 3-keto carbofuran, 3-hydroxycarbofuran phenol, 3-hydroxycarbofuran and carbofuran phenol, were present in about equal amount. The nature of the excreted residues from the other insecticides was not determined.

^{14}C-Residue in Tissues - Ninety min after smoking, ^{14}C-residues from all the compounds were detected in the liver, kidney, muscle, fat, spleen, brain, ovary and uterus. The persistence of the residues, however, was quite variable. After 4 days, only the fat contained residues greater than 0.2% of the total inhaled ^{14}C-pyrolysis products derived from the organophosphates. At this time, the total body burden amounted to 1.0% of the inhaled activity. ^{14}C-Carbaryl residues remaining after 4 days amounted to 1.6% of the inhaled radiocarbon, 75% of which was in the fat, kidney, and liver. Carbofuran radiocarbon, 0.6%, was found only in the liver and blood.

While ^{14}C-DDT residues declined in the liver, kidney, muscle and other organs during the 4 days, concentrations in the fat increased to over 3 times the level observed at 90 min. The percentage of DDT pyrolysis products remaining in the body 4 days after exposure was 48.7% of the inhaled radioactive residues.

Carbofuran Pyrolyses - The technique of mechanical pyrolysis, i.e. the use of pyrolysis chambers, etc., has been extensively used in efforts to determine the chemical processes which occur during tobacco combustion. While the dangers of extrapolating the pyrolysis results to processes actually occurring during smoking are well understood, discrepancies may be even more apparent when considering the fate of volatile materials on the burned tobacco. This issue was evaluated in the present study by comparing the pyrolytic behavior of carbofuran in a "conventional" pyrolysis apparatus, i.e. a vertical combustion tube encased in a fixed position oven, to that using a horizontal pyrolysis apparatus recently developed. In the latter, the sample is evenly distributed inside a horizontal quartz tube and heated by a movable, variable temperature oven. In contrast to the "conventional" apparatus, the slow movement of the oven along the tube results in a burning process similar to that of a burning cone of a cigarette.

For pyrolysis using the horizontal quartz tube, 5.5 g of carbofuran was evenly distributed along 80 cm of its length. After preheating to the desired temperature, ca. 1 hr, the oven was set in motion, resulting in a 5.1 g of brown semisolids collected at the end of the tube and in the first cold trap. Trituration of the pyrolyzate with cold 1:1 hexane-benzene yielded 1.6 g of crystalline carbofuran, confirmed by melting point and tlc. The filtrate was diluted with 100 ml of benzene and quickly extracted 3 times with 20 ml of cold 5% sodium hydroxide. The organic phase was dried with sodium sulfate and concentrated to yield an additional 380 mg of the intact insecticide. The basic extract was acidified and repeatedly extracted with ether. The organic layers were combined, dried and concentrated under vacuum to yield 2.1 g of an oily residue, most of which was the phenol of carbofuran. A minor component ($<1\%$) of the phenolic fraction, collected by preparative glc, was tentatively identified as 2-methyl-2-benzofuranol; ir($CHCl_3$) 2.90, 6.21 and 6.93 μ; nmr ($CDCl_3$)δ; 2.38

(s, 3p), 6.35 (s, 1p) and 6.6 to 7.1 (m, 4p); ms 70eV m/e, 148 (P), 131, 119, 91 and 65. The infra-red spectrum of the evolved gases showed characteristic bands of a mixture of methyl isocyanate, methane, and carbon dioxide.

With the vertical pyrolysis apparatus, the pyrolysis tube was heated to 783°C, and 8.6 g of carbofuran introduced into the top of the column over a 30 min period. At the completion of the experiment, the apparatus was cooled to room temperature and 2 g of material which had solidified before reaching the cold traps were triturated with hexane-benzene to yield 1.2 g of parent compound. The contents of the traps, 4.3 g, were dissolved in chloroform and quickly extracted 3 times with 50 ml of ice cold 5% sodium hydroxide. The resulting chloroform phase was dried with magnesium sulfate, concentrated under vacuum and the solid residue washed with hexane-benzene and filtered to furnish 2.2 g of carbofuran. The filtrate was concentrated to 680 mg of oil that was composed of at least 46 compounds as indicated by glc. GC/MS analysis showed the presence of naphthalene, benzofuran, biphenyl and anthracene in the neutral fraction.

The aqueous layer was acidified and extracted with ether and the extracts combined, dried, and concentrated under vacuum to give 1.7 g of dark oil. Glc analysis on OV-101 and EGSSX columns showed that about 90% of the material consisted of two major components whose spectral and chromatographic characteristics were identical to the phenol of carbofuran and to 2-methyl-7-benzofuranol. The balance of the material was composed of at least three unknowns. Gaseous pyrolysis products consisted of methane, carbon monoxide, carbon dioxide and methyl isocyanate.

Results of the carbofuran pyrolysis experiments clearly show that the horizontal combustion apparatus more closely approximated the smoking process than did the vertical unit. The latter system resulted in numerous carbofuran derivatives which were not detected in the mainstream smoke of cigarettes, and would suggest a far more complicated situation than actually exists. Since pyrolysis of pesticides in the absence of tobacco greatly facilitates isolation and identification of their pyrolysis products, such a process is very desirable. However, great care must be taken to assure that the nature of the products is the same as that in tobacco smoke. It appears that this can be largely accomplished by using the horizontal pyrolysis apparatus.

SCREENING OF PESTICIDES FOR MUTAGENIC POTENTIAL USING THE AMES ASSAY

A wide variety of test systems with differing degrees of simplicity and sensitivity have been developed to detect the mutagenic and carcinogenic potential of chemicals, tobacco smoke fractions, and extracts of airborne pollutants. The methods involve use of cultured cells, microorganisms, plants, or intact animals, and have been recently reviewed by Stolz *et al.* (1974) and Epstein and Legator (1971).

Ames *et al.* (1973 a,b) have introduced an assay for the detection of mutagens using histidine-requiring mutants of *Salmonella typhimurium*. The assay is quite sensitive to detect point mutations of both the frameshift and base-pair substitution type, is easy to perform, and includes a capacity to approximate mammalian metabolism by the addition of a rat liver homogenate fraction. In the present study, we have employed the Ames assay procedure to evaluate the mutagenic potential of several pesticides which have been used extensively for the control of crop and animal pests.

Materials and Methods

Chemicals - Nitrosocarbaryl (N-nitroso-N-methyl-1-naphthylcarbamate) was prepared according to procedures outlined by Elespuru *et al.* (1974). The structure was confirmed by a mass spectral analysis (70 eV), which showed a parent ion at m/e 230 and fragments at m/e 143 (naphthoxyl), m/e 127 (naphthyl), and m/e 115, as previously reported. Purity was confirmed by silica gel thin-layer chromatography in three solvent systems: 9:1 carbontetrachloride:ether; 1:1 Chloroform:ether; and 1:1 ethyl acetate:ether. Aroclor 1254, DDT, DDE, dieldrin,heptachlor, heptachlor epoxide, carbaryl, diazinon, linuron, and captan were obtained in a pure form from stock of standards used in residue analysis. N-methyl--N-nitro-N'-nitrosoguanidine (MNNG) and 2-acetylaminofluorene (AAF) were purchased from Aldrich Chemical Company. Sodium phenobarbital was obtained from the W. A. Butler Company. Benz[a]anthracene, 9-aminoacridine, 7-iodomethyl-12-methylbenz[a]anthracene, 7,12-dimethylbenz[a]anthracene (DMBA), DMBA dialdehyde, and 6-chloromethylbenzo[a]pyrene were supplied by Dr. James Flesher, Department of Pharmacology, University of Kentucky.

Bacterial System - The bacterial strains (TA1535, TA1536, TA1537, TA1538) were acquired from Dr. Ames (Biochemistry Department, University of California, Berkeley). These are histidine-requiring mutants which, in addition, lack an excision repair system, and the lipopolysaccharide barrier making them more sensitive and permeable to foreign compounds. Strain TA1535 (Ames *et al.* 1973a) is a base-pair substitution mutant, while the other three are frameshift mutants.

The agar medium was prepared according to Ames *et al.* (1973a) as was the "S-9" liver preparation. Induction of the liver enzyme system was accomplished by providing 200 g Sprague-Dawley male rats with drinking water containing 0.1% phenobarbital for seven days, or by injecting the rats i.p. with Arochlor 1254 at 200 mg/kg 5 days before sacrifice.

The test methods used were exactly as described by Ames *et al.* (1973b). All the compounds tested were dissolved in dimethylsulfoxide (DMSO) in concentrations varying from 1 to 10 mg/ml. Up to 0.5 ml of DMSO solution was added with the soft agar to each plate, as was 0.1 ml of bacterial tester strain culture (10^9 bacteria/ml estimated by measuring turbidity on a Klett spectrophotometer). These plates were incubated for 48 hours at 37°C and data quantitated by counting the number of colonies per plate. Each colony represents a reverse mutation from the histidine-dependent to the histidine-independent form. 2-Acetylaminofluorene and N-methyl-N-nitro-N'-nitrosoguanidine were used as active standards or positive controls in the prescribed manner (Ames *et al.* 1973b). Cellular toxicity was determined by mixing each compound with the tester strain as described above, but with the top agar containing an excess of histidine (3 μmoles/plate). The plates for toxicity determinations were incubated for 24 hours at 37°C and evaluated for growth inhibition by comparison with control plates.

The water soluble metabolites from bean plants each treated with 500 μg of carbaryl were tested in the Ames assay. Since the injection to the bean plants contained a small amount of carbaryl ring [^{14}C] as a tracer, the carbaryl equivalents in the water soluble metabolites were determined by radioassay. The water soluble metabolites of carbaryl and the water fraction from control plants were lyophilized to dryness. The carbaryl water soluble metabolites were tested in a solution of 1 mg equivalents of carbaryl to 1 ml DMSO at levels up to 500 μg equivalents of carbaryl per plate. Equal volumes of the control plant residue were tested for mutagenicity and toxicity. Samples of carbaryl ring [^{14}C] were tested at levels up to 100,000 dpm (twice the radioactive content of the highest level of carbaryl water soluble metabolites). Carbaryl itself was tested up to 1000 μg per plate.

DDE, dieldrin, hpetachlor, heptachlor epoxide, and diazinon were tested from 5 to 1000 μg per plate, and DDT was tested from 5 to 2500 μg per plate. Linuron and captan were tested from 1 to 200 μg per plate.

Results and Discussion

The Ames assay is an *in vitro* testing system which uses histidine requiring mutants of *Salmonella typhimurium* to screen chemicals for potential *in vivo* mutagenicity. If the chemical being tested causes a mutation in a cell of one of the strains (TA1535, TA1536, TA1537, or TA1538) at a point in the gene responsible for histidine dependence, such that the cell becomes histidine independent, then a reverse mutation has taken place. This cell will grow into a revertant colony on histidine deficient agar. The number of these colonies is a measurement of the potential mutagenicity of the compound *in vivo*. The higher the number is, the greater the potential.

In order to obtain an appreciation for the amount of spontaneous revertant colonies to expect, many plates were assayed with only the ingredients of the nutrient agars and 0.5 ml dimethylsulfoxide, the solvent used to dissolve the test compounds. The results of this undertaking are illustrated in Table 27. The mean spontaneous reversion frequencies were 10.7 with 1535, 0.6 with 1536, 10.2 for 1537, and 17.3 for 1538. Another series of plates were assayed with the same ingredients plus a rat liver homogenate (S-9 RLH). The mean spontaneous reversion frequencies in this series were 10.3 for 1535, 2.7 for 1536, 15.0 for 1537, and 32.1 for 1538. Along with the mean spontaneous reversion frequencies listed in Table 27 are the standard deviations of these means. The considerable variation observed could have been narrowed by two procedures: 1) expressing the reversion frequency in terms of number per million viable bacteria, which would require a dilution series plate count on each date of testing; and 2) standardizing the stock cultures each time they are grown (every seven days) to 10^9 bacteria per ml by a dilution series plate count, instead of estimating this on a spectrophotometer. However, these operations were considered unnecessary for the purpose of screening large numbers of chemicals for mutagenic potential. Therefore, it was decided that in order for a compound to be declared mutagenic in this test system, it must demonstrate a reproducible dose response curve with a peak reversion level well above several standard deviations from the spontaneous reversion frequency.

Table 28 is a compilation of mutagens, carcinogens, and carcinogen analogs, along with results of tests in the Ames assay. There was a wide variety of activity demonstrated by these compounds, but the known carcinogens, 2-acetylaminofluorene (AAF), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), 7-iodomethyl-12-methyl-benz[a]anthracene, 7,12-dimethylbenz[a]anthracene, and 6-chloromethyl benzo[a]pyrene, were at least mildly active. The carcinogen benz[a]anthracene was not active in the four tester strains, and neither was the DMBA dialdehyde, a noncarcinogen. These data illustrated certain characteristics of the Ames assay. For example, with AAF and DMBA no mutagenic activity was manifested until

Table 27. Spontaneous reversion frequency in TA1535 series of *S. typhimurium* with (+) and without (-) rat liver homogenate.

	Spontaneous reversion per 10^8 bacteria/strain							
	1535		1536		1537		1538	
Parameters	-	+	-	+	-		-	+
Mean	10.7	10.3	0.6	2.7	10.2	15.0	17.3	32.1
Standard deviation	±7.0	±5.7	±1.1	±3.5	±5.0	±8.6	±6.7	±11.1
Range	1-35	1-28	0-5	0-15	1-22	2-43	1-43	15-56
Number of samples	49	38	33	28	49	43	59	49

Table 28. Mutagenic activity of known mutagens, carcinogens, and carcinogen analogs in *S. typhimurium* - TA1535 series.

Compound	µg/plate ±RLH*	Revertant colonies per plate/strain			
2-acetylamino-fluorene (AAF)	50-	22(16)	1(1)	12(18)	28(32)
	50+	18(21)	0(1)	88(29)	5611(44)
N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	2-	1812(14)	0(0)	10(12)	18(29)
	5-	10560(20)	--	7(17)	13(22)
9-aminoacridine	100-	11(10)	--	900(12)	11(18)
benz[a]anthracene	100+	6(14)	--	18(18)	21(38)
7-iodomethyl-12-methylbenz[a]-anthracene	10-	8(10)	1(0)	198(12)	480(18)
	10+	8(14)	3(1)	268(18)	456(38)
DMBA-dialdehyde	100-	8(10)	0(0)	6(12)	10(18)
	100+	15(14)	14(7)	12(18)	20(38)
7,12-dimethyl-benz-[a]anthracene (DMBA)	25-	--	--	17(18)	36(38)
	25+	--	--	63(18)	90(38)
6-chloromethyl-benzo[a]pyrene	25-	2(5)	0(0)	3(8)	1280(16)
	25+	6(8)	1(2)	10(15)	1600(32)

* with (+) or without (-) rat liver homogenate

( ) spontaneous reversion frequency for each test the S-9 rat liver homogenate fraction was added. The capacity of mammalian metabolism enables this test system to detect many carcinogens as mutagens which, otherwise, would not be detected. On the other hand, MNNG and 9-aminoacridine were quite active without the liver homogenate. It is believed that these two chemicals require no *in vivo* biochemical activation to express their mutagenic properties (Miller 1970). These data supported the claim that the Ames assay was a good screening test for potential mutagenicity and carcinogenicity (Ames *et al.* 1973b).

Table 29 contains the results of tests using the Ames assay on four pesticides purported to be potential human carcinogens, DDT, dieldrin, heptachlor, and captan. Also tested were DDE, heptachlor epoxide, diazinon, carbaryl, nitrosocarbaryl, and linuron, all pesticides or pesticide derivatives, which have demonstrated some mutagenic manifestation in other assay systems (Vaarama 1947, Amer 1965, Markaryon 1967, Wuu and Grant 1967, Epstein and Shafner 1968, Kaszubiak 1968, Legator *et al.* 1969, Elespuru *et al.* 1974). The control values represent the spontaneous reversion frequencies of the tester strains both with and without rat liver homogenate. Where no activity was observed, only the highest dose tested or the maximum tolerated concentration (the highest concentration which did not appreciably inhibit growth) is listed. However, all the compounds were evaluated at concentrations as low as 5 µg/plate with each successive level increased by a factor of two up to the highest level. The toxicity data were useful in determining the concentrations that gave maximum reversion with a minimum of growth inhibition, an influence that would obviously affect the dose-response correlation.

The results revealed two active compounds, captan and nitrosocarbaryl. Both are active as base-pair substitution mutagens as evidenced by their ability to back mutate the 1535 strain. According to Ames *et al.* (1973a), the exact base-pair changes which revert this strain are not clear, but it is suspected that most, if not all, of the six possible base-pair substitutions can be detected.

Table 29. Mutagenicity in *S. typhimurium* - TA1535 series.

Compound	μg/plate ±RLH*	Revertant colonies per plate/strain				Toxic level μg ±RLH*
		1535	1536	1537	1538	
DDT	2500-	6(8)	0(0)	10(15)	22(19)	>2500-
	2500+	15(17)	3(8)	45(43)	69(56)	>2500+
DDE	1000-	4(7)	0(1)	10(13)	17(13)	>2500-
	1000+	18(17)	3(8)	22(43)	69(56)	>1000+
Dieldrin	1000-	3(8)	0(1)	8(11)	16(18)	>2500-
	1000+	17(17)	18(8)	37(43)	65(56)	>1000+
Heptachlor	1000-	4(10)	1(0)	16(11)	12(12)	>2500-
	1000+	8(17)	8(8)	41(43)	53(56)	>1000+
Heptachlor	1000-	10(13)	0(0)	10(13)	14(10)	>2500-
epoxide	1000+	15(17)	7(8)	55(43)	65(56)	>1000+
Diazinon	1000-	12(10)	1(0)	9(11)	13(13)	>1000-
	1000+	22(17)	7(2)	3(18)	13(33)	>1000+
Carbaryl	1000-	19(9)	0(2)	19(11)	22(23)	>2500-
	1000+	9(17)	10(8)	33(43)	51(56)	>1000+
Nitrosocarbaryl	0.5-	51(8)				100-
	5-	2976(8)				
	50-	2932(8)	0(0)	92(15)	96(20)	
	100-	3(8)	0(0)	115(15)	52(20)	
	5+	57(10)				>1000+
	50+	870(17)	2(2)	21(8)	56(56)	
	300+	5252(17)	0(2)	27(8)	61(56)	
	500+	3892(17)	2(2)	37(8)	42(56)	
	1000+	1268(17)		45(15)	38(56)	
Linuron	25-	8(8)	2(0)	4(22)	13(28)	50-
	200+	11(13)	1(1)	8(17)	40(46)	∿ 400+

Table 29. Continued.

Compound	µg/plate ±RLH*	Revertant colonies per plate/strain 1535	1536	1537	1538	Toxic level µg ±RLH*
Captan	2.5-	100(7)				
	10-	248(10)	0(1)	335(7)	19(32)	100-
	25-	320(10)	2(1)	47(7)	11(32)	
	50-	596(10)	0(1)	0(7)	0(32)	
	25+	117(14)	2(1)	148(9)	32(32)	∿400+
	50+	231(14)	0(1)	288(9)	51(32)	
	200+	221(16)		78(2)	34(32)	

* RLH=S-9 rat liver homogenate

( ) Spontaneous reversion rate for each test

Nitrosocarbaryl was extremely potent, demonstrating increased reversion at 0.5 μg level in the base substitution revertible strain 1535. This compound has demonstrated mutagenic characteristics in two other bacteria, *Escherichia coli* and *Haemophilus influenzae* (Elespuru *et al.* 1974). Beattie and Kimball (1974) reported that mutagenesis induced in *H. influenzae* by nitrosocarbaryl consists of both replication-dependent and replication-independent components. Our results with strain 1537 and 1538 indicate that nitrosocarbaryl may be slightly active as a frameshift mutagen at higher concentrations. TA1538 is particularly sensitive to the polycyclic aromatic hydrocarbons due to a specific "hot spot" in the DNA (Ames *et al.* 1973a). Presence of the "S-9" liver homogenate decreased the mutagenic action of nitrosocarbaryl, thus indicating that activation was not required.

Four series of tests on nitrosocarbaryl in TA1535 representing four different stock cultures of that strain varying in age from two to seven days were combined for a regression analysis. It was found that the age of the culture had no significant contribution to the variability of the system. Utilizing all the data generated in this study with nitrosocarbaryl, a correlated T-test showed that the 1 μg level of nitrosocarbaryl without rat liver homogenate and the 50 μg level with rat liver homogenate were the concentration levels showing the least significant difference ($p<.01$) from the spontaneous reversion frequency.

Based on the results with nitrosocarbaryl and assuming that the variability is inherent within the method regardless of the activity of the compound, a hypothetical compound tested twice would need to demonstrate a reversion mean at the peak response level of at least 135 without rat liver homogenate, and at least 320 with rat liver homogenate in order to conclude that the compound was mutagenic in strain 1535 ($P=.05$). This conservative approach is necessary to eliminate the false positives which could occur if one attempted to apply the standard T-test. However, the strict adherence to numbers obtained from such a statistical analysis is not recommended until a large number of active compounds are studied under similar conditions. Even then the results should be viewed only as a guideline by the researcher.

Captan was much less potent than nitrosocarbaryl, but also was active as a frameshift mutagen in strain 1537. Similarly, it seemed to act directly since the liver homogenate decreased its activity. However, activation via the metabolic pathways of the bacteria cannot be ruled out completely for captan or nitrosocarbaryl. Several *in vitro* studies (Epstein and Shafner 1968, Legator *et al.* 1969) have shown captan to be mutagenic, while results of other *in vivo* studies fail to correlate with these findings (Kennedy *et al.* 1975). This may be due to captan's short half life in serum (Epstein and Legator 1971).

The other eight compounds were negative in this system. Unless toxicity was noted, they were tested in concentrations from as low as 1 to 2500 µg/plate. Ideally, compounds should be tested up to levels of growth inhibition as was done with linuron, but at the highest levels shown in Table 29, precipitation was seen with the chlorinated hydrocarbons and with carbaryl. The use of DDT, dieldrin and heptachlor has been banned or severely limited by the Environmental Protection Agency because of their carcinogenic activity in experimental mammals. A lack of activity of these chemicals in the Ames assay may be indicative of its insensitivity to certain types of mutagens and/or carcinogens, or of the inconclusiveness of studies conducted in higher organisms.

The carbaryl water soluble metabolites from bean plants treated with 500 µg of carbaryl were tested in the Ames assay. The results are shown in Table 30. These metabolites were not mutagenic at levels up to 500 µg equivalents of carbaryl. Higher levels were not possible due to the limited amount of test material. Carbaryl, itself, was not mutagenic at levels up to 1000 µg/plate. It is of interest to note the inhibition of growth seen at the 500 µg level (concentration = 1 mg equivalent of carbaryl per ml) in the carbaryl water soluble metabolite plates, since carbaryl itself was not toxic to the bacteria at 2500 µg/plate. However, an equivalent amount of control plant water soluble material was also

Table 30. Mutagenicity screening of carbaryl bean plant water soluble metabolites in *S. typhimurium* - TA1535 series.

Compound	Treatment per plate ±RLH[a]	Revertant colonies per plate			Toxic level[b] ±RLH[a]
		1535	1537	1538	
Carbaryl bean plant water soluble metabolites	10 μg[c] -	4(11)	5(8)	9(10)	500 μg[c] -
	50 μg -	10	10	8	
	100 μg -	5	8	10	
	500 μg -	1	--	0	
	10 μg +	10(14)	33(22)	15(14)	500 μg +
	50 μg +	9	38	16	
	100 μg +	3	10	21	
	500 μg +	0	--	--	
Control bean plant water soluble fraction	10 μl[d] -	6(10)	8(6)	7(7)	500 μl[d] -
	50 μl -	4	6	9	
	100 μl -	5	8	9	
	500 μl -	7	--	5	
	10 μl +	9(10)	9(6)	26(21)	
	50 μl +	10	5	20	
	100 μl +	14	16	23	
	500 μl +	15	--	--	
Naphthyl[^{14}C]-carbaryl	50,000 dpm[e] -	5(4)	3(5)	11(10)	
	100,000 dpm[f] -	3	7	10	
	50,000 dpm +	12(7)	10(5)	15(16)	
	100,000 dpm +	7	5	16	
Carbaryl	1000 μg -	19(9)	19(11)	22(23)	>2500 -
	1000 μg +	9(17)	33(43)	51(56)	>1000 +

( ) Spontaneous reversion frequency for each test series.

[a] With (+) and without (-) rat liver homogenate.

[b] Inhibition of bacterial growth was used as an indication of toxicity.

[c] μg equivalents of carbaryl as determined by radioactive content.

[d] μl equivalents of the control plant water soluble/plate.

[e] .23 μg carbaryl.

[f] .46 μg carbaryl (up to twice the ^{14}C content of the carbaryl plant water soluble plates).

toxic at the 500 μl/plate level, indicating some inherent toxicity of this plant fraction not related to any carbaryl metabolites. The ring [^{14}C] carbaryl (specific activity = 19.6 mCi/mM) was included in this study as a check on any mutagenicity due to the low radioactivity present in the carbaryl water soluble metabolites tested. This radioactive preparation was tested at dpm levels up to twice the amount present in the highest level of the carbaryl water soluble metabolites (50,000 dpm). No mutagenicity was demonstrated.

The fact that carbaryl water soluble metabolites were negative in this test system is noteworthy. As with many carbamate insecticides, a large portion of carbaryl plant insecticide residues are of a water soluble nature, thus resulting in long term, low level exposure to the human population. Under these conditions carcinogenicity, mutagenicity, and teratogenicity are toxic effects of greatest concern to regulatory agencies. Although none of these effects have been implicated due to the ingestion of any pesticide conjugate (which the water soluble metabolites are comprised of), the possibility of chronic toxic effects does exist (Dorough 1976). The sulfate ester of N-hydroxy-2-acetylaminofluorene is a good example (DeBaun *et al.* 1966). This metabolite is a more potent carcinogen than the parent compound, 2-AAF.

Given the chemical structures and the theory on alkylating agents pertaining to their mechanism of mutagenesis, the results obtained in these experiments are somewhat predictable. Both nitrosocarbaryl and captan have good "leaving groups", thus they could be expected to interact with cellular nucleophiles such as DNA and proteins. Conversely, the other compounds have no such easily identifiable group. Of course, this does not rule out their being active by other mechanisms.

As part of the report prepared for the Mrak Commission (Secretary's Commission on Pesticides and Their Relationship to Environmental Health) in 1970, Epstein and Legator (1971) recommended a program for mutagenesis testing of pesticides. This included three mammalian test systems (the dominant lethal, host mediated, and *in vivo* cytogenetic), and several ancillary microbial systems for detecting both single nucleotide changes and effects involving more than one gene. Results reported herein indicate that the Ames assay would be a valuable asset to such a battery of tests, because of its sensitivity, ease of performance, and mammalian metabolizing capacity. Not only would the assay aid in evaluating the mutagenic potential of a pesticide, but also its carcinogenic hazard, since a very high correlation exists among known carcinogens and their ability to back mutate the TA1535 series of *S. typhimurium* (Ames *et al.* 1973b).

Any conclusions based solely on the Ames assay, though, are not the final answer. One need only scan the results in Table 29 relative to the pesticides screened in the Ames assay to realize that problems exist in detecting weak or potential carcinogens, such as DDT, heptachlor, and dieldrin. Even the known carcinogen benz[a]anthracene was negative with the four tester strains shown in Table 28. However, recently introduced strains of *S. typhimurium* have shown this compound as an active mutagen (McCann *et al.* 1975). A priority search is under way in the laboratory of Ames to find *S. tyhpimurium* strains that are sensitive to the chlorinated insecticides suspected to be carcinogens. The possibility exists, though, that these chemicals are not mutagens. On the other hand, captan has demonstrated no evidence of being a genetic hazard in mammalian species, but it is active in several bacterial strains including those of Ames (Kennedy *et al.* 1975, McCann *et al.* 1975). Obviously, caution should be used in extrapolating effects observed in bacteria to higher organisms.

METABOLISM OF CARBARYL IN THE AMES MUTAGENIC SCREENING SYSTEM

The Ames mutagenic assay system has become a valuable tool to screen chemicals for possible mutagenic/carcinogenic activity. Success of this assay procedure can be partially attributed to the addition of S-9 rat liver homogenate (RLH) which is assumed to simulate mammalian metabolism. The metabolic activity of the S-9 RLH has not been fully demonstrated and it is possible that the agar-bacteria-liver enzyme combination may either (a) produce none of the metabolites formed in animals, or (b) produce active metabolites that are not common to the _in vivo_ animal system. The inability of the Ames system to detect 10% of the 175 carcinogens tested (McCann et al. 1975) may be attributed to the failure of the system to metabolically activate the compounds. Such possibilities have prompted this study which has analyzed the metabolic capabilities of the Ames system on the test compound carbaryl.

Materials and Methods

Chemicals - Carbaryl-1-naphthyl-^{14}C (19.6 μCi/mmole), carbaryl-carbonyl-^{14}C (5.38 μCi/mmole), 4-hydroxy carbaryl, 5-hydroxy carbaryl, N-hydroxymethyl carbaryl, 5,6-dihydrodihydroxy carbaryl, 5,6-dihydrodihydroxy naphthol, and α-naphthol were obtained from laboratory stocks. Purity of each compound was confirmed by thin-layer chromatography (tlc).

Test System - Experimental conditions for the Ames test are described by Marshall _et al_. (1976). Because of interfering properties of dimethyl sulfoxide with tlc analysis, carbaryl was added as an acetone solution not to exceed 20 μl of acetone per plate. The experiment was designed to evaluate the metabolic capabilities of each major constituent of the Ames system. This necessitated the analysis of possible chemical degradation occurring in the complete top agar (Treatment I), microbial metabolism in the inoculated top agar (Treatment II), "mammalian" metabolism in the S-9 RLH-top agar complex (Treatment III), and a combination of all of these (Treatment IV). Duplicate plates, representing each treatment and various incubation periods, were prepared and incubated at 37°C.

After incubation the plates were immediately frozen at -20°C to stop the metabolic reactions. Each plate contained approximately 112,000 dpm (0.52 μg) of carbaryl-1-naphthyl-^{14}C. The test organism used throughout this study was *Salmonella typhimurium* strain TA1535, a base-pair substitution mutant. Since carbaryl could not promote growth of the histidine-requiring mutants, excess histidine (0.63 μg/plate) was added to additional plates from treatments II and IV to evaluate the metabolism of carbaryl by actively growing organisms. Zero-hour, control plates from treatments III and IV received inactivated (boiled) S-9 RLH.

The metabolism of carbaryl-carbonyl-^{14}C was used as an aid in ascertaining the identity of some of the carbaryl metabolites. Comparison of metabolites from both labeling patterns indicated which metabolites retained the carbonyl carbon.

Extraction Procedure - The contents of each petri plate were blended with 25 ml of distilled water and 25 ml of ethyl acetate using a Lourdes blender. The mixture was centrifuged for 30 min at 3300 x g, and the liquid was decanted into a 125-ml separatory funnel. The organic phase was collected and the aqueous and agar phases were re-extracted with an additional 25 ml of ethyl acetate. Aliquots of each fraction were quantitated by liquid scintillation to determine the distribution of radiocarbon. Each sample was stored at -10°C until further analysis.

Thin-Layer Chromatography - Organo-soluble metabolites were analyzed by one-dimensional thin-layer chromatography using a methylene chloride: ethyl acetate (1:1 v/v) solvent system. Radioautograms were prepared by exposing tlc plates to X-ray film for a minimum of seven days. Radioactive bands were scraped and quantitatied by liquid scintillation counting.

Results and Discussion

The Ames test is routinely conducted both with and without the addition of S-9 RLH. This allows the detection of direct-acting mutagens or active metabolites of the test compound. Two basic assumptions must be made with this type of procedure. The first assumption is that neither the chemical environment nor the microorganisms have the ability to detoxify the active product to prevent a positive response. Secondly, it must be assumed that the S-9 RLH is representative of mammalian metabolism. These assumptions must be questioned particularly in cases where the Ames test has failed to detect known carcinogens.

Although carbaryl is not a mutagen/carcinogen, its susceptability to microbial and mammalian metabolism makes it an excellent reference compound to test the metabolic activity within the Ames system. Distribution data in Table 31 indicated the rate of carbaryl conjugation occurring in the Ames system. The insignificant percentage of water soluble products detected in plates from Treatment I suggests that the chemical environment, consisting of agar, histidine, biotin, and glucose, had little effect on carbaryl metabolism. Similar results were obtained with inoculated plates from Treatment II, suggesting a lack of metabolism by the *Salmonella typhimurium* mutants. Analysis of the organosoluble products revealed that 77% of the parent compound still remained even after 48 hours of incubation (Table 32). Since no major metabolites were detected, the 22% loss of carbaryl was believed to result from a general loss of product during tlc analysis. The addition of excess histidine to enhance the microbial growth resulted in a slight accumulation (<1%) of N-hydroxymethyl carbaryl, but this was considered insignificant in relation to the total concentration of carbaryl. These results indicate that the microorganisms had little effect upon the metabolism of carbaryl.

The metabolic ability of the S-9 RLH is quite apparent from the distribution data (Treatment III) presented in Table 31 Within one hour of

Table 31. DISTRIBUTION OF RADIOCARBON AFTER INCUBATING CARBARYL-1-NAPHTHYL-^{14}C WITH VARIOUS COMPONENTS OF THE AMES ASSAY SYSTEM

Treatment[a] and fraction	% per fraction/hr of incubation						
	0	1	3	6	12	24	48
I.							
Organo-soluble	100.0	100.0	100.0	83.3	87.3	80.4	80.7
Water soluble	0.1	0.1	0.1	0.3	0.8	2.6	7.2
Bound (agar)	0.1	0.1	0.1	0.2	0.5	2.3	5.8
Total	100.0	100.0	100.0	83.8	88.6	85.3	93.7
II.							
Organo-soluble	97.8	96.7	100.0	82.2	94.1	89.2	86.6
Water soluble	0.1	0.1	0.1	0.3	1.7	2.1	5.3
Bound (agar)	0.1	0.1	0.1	0.2	0.5	1.9	4.8
Total	98.0	96.9	100.0	82.7	96.3	93.2	96.7
III.							
Organo-soluble	95.3	49.7	47.6	44.1	41.8	45.9	45.9
Water soluble	0.1	24.3	27.0	28.0	26.9	27.1	17.0
Bound (agar)	0.1	15.4	17.8	16.9	19.1	20.1	18.0
Total	95.5	89.4	92.4	89.0	87.8	93.1	80.9
IV.							
Organo-soluble	95.9	50.8	45.4	37.6	38.4	39.6	42.7
Water soluble	0.0	24.5	24.7	26.7	26.3	30.1	27.9
Bound (agar	0.0	14.5	17.3	17.7	20.9	20.6	16.9
Total	95.9	89.8	87.4	82.0	85.6	90.3	87.5

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[a] Each treatment conducted under conditions of the Ames test. Petri plates contained the solid bottom agar plus the:

(I) complete top agar consisting of soft agar, histidine, and biotin;
(II) complete top agar and 10^8 mutants;
(III) complete top agar and the S-9 rat liver homogenate;
(IV) complete top agar, the mutants, and the S-9 rat liver homogenate.

Table 32. CARBARYL-1-NAPHTHYL-^{14}C REMAINING AFTER INCUBATION WITH VARIOUS COMPONENTS OF THE AMES ASSAY SYSTEM

	% as carbaryl/hr of incubation						
Treatment[a]	0	1	3	6	12	24	48
I	99.0	NA	NA	NA	87.3	NA	77.9
II	95.9	NA	NA	NA	92.0	NA	82.7
III	93.3	8.7	2.4	0.8	2.5	0.9	4.0
IV	94.0	10.9	2.8	0.8	1.5	0.4	1.1

[a] Each treatment conducted under conditions of the Ames test. Petri plates contained the solid bottom agar plus the:
(I) complete top agar consisting of soft agar, histidine, and biotin;
(II) complete top agar and 10^8 mutants;
(III) complete top agar and the S-9 rat liver homogenate;
(IV) complete top agar, the mutants, and the S-9 rat liver homogenate.

NA = not analyzed

incubation, approximately 25% of carbaryl was converted to water soluble products and 15% to agar-bound materials. The distribution of radioactivity among the phases remained fairly constant after one hour. This demonstrated that the enzymes had lost most of their activity within the first hour of incubation at 37°C. However, analysis of the organo-soluble products from plates of Treatment III suggested that the S-9 fraction was slightly active for up to six hours in that the concentration of carbaryl continued to decline during this period (Table 33). This agrees with the contention of Ames that the solid agar tends to prolong the activity of the liver enzymes, although it is clear that, as with other *in vitro* RLH systems, the major activity is evidenced shortly after the mixing of enzyme and substrate. The degree of activity of the S-9 RLH is indicated by the production of nine metabolites in the organic phase.

Tentative identification of the metabolites was based upon cochromatography with known reference standards and comparisons of metabolites using two different labeling patterns. Five of the metabolites were identified as follows: 5,6-dihydrodihydroxy carbaryl, 5,6-dihydrodihydroxy naphthol, N-hydroxymethyl carbaryl, 4-hydroxy carbaryl, and 5-hydroxy carbaryl. The identity of the remaining products could not be confirmed because of their low yield. Unknown 4 cochromatographed with α-naphthol but the carbonyl labeled experiment revealed that this product retained the carbonyl carbon as did the other unidentified metabolites. Further characterization of the unknown metabolites has not been attempted since it was not the intent of this study to completely characterize carbaryl metabolism, but to evaluate the metabolic activity of the S-9 RLH.

Results of this study show that the homogenate was capable of rapidly metabolizing carbaryl primarily via oxidative and conjugative routes. The identified free products were all oxidative metabolites and were probably metabolized further to glucuronides and sulfate conjugates as in other mammalian systems. The identity of these conjugates was not verified but their presence was indicated by the production of water

Table 33. NATURE OF THE RADIOCARBON IN THE ORGANO-SOLUBLE FRACTION RESULTING FROM THE INCUBATION OF CARBARYL-1-NAPHTHYL-^{14}C WITH ALL COMPONENTS OF THE AMES SYSTEM EXCEPT THE MICROORGANISMS, TREATMENT III[a]

Metabolites resolved by TLC	% of ^{14}C in organo-soluble fraction/hr of incubation							
	R_f	0	1	3	6	12	24	48
Unknown 1	0.00	0.2	8.3	9.1	7.1	10.6	10.5	14.1
Unknown 2	0.04	0.0	1.7	2.5	2.5	2.8	2.9	3.9
5,6-dihydro-dihydroxy carbaryl	0.14	0.0	35.6	47.4	50.7	45.7	55.0	32.3
5,6-dihydro dihydroxy naphthol	0.30	0.0	2.8	4.5	7.2	8.9	15.3	19.5
Unknown 3	0.39	0.0	2.7	4.9	6.1	4.1	3.9	3.5
N-hydroxymethyl carbaryl	0.50	0.0	9.6	6.5	3.7	4.1	1.3	2.1
4-hydroxy carbaryl	0.68	0.0	6.0	7.2	9.8	6.4	2.8	3.5
5-hydroxy carbaryl	0.79	0.0	6.6	8.3	8.1	5.9	2.5	3.6
Carbaryl	0.90	99.0	21.0	6.1	2.2	7.1	2.3	10.6
Unknown 4	0.98	0.8	5.7	3.3	2.6	4.6	3.6	6.9

[a] Petri plates containing the bottom agar, the complete top atar, and the S-9 rat liver homogenate of the Ames system, but without *S. typhimurium*.

soluble products. Evidence obtained thus far has demonstrated that the S-9 RLH is an efficient *in vitro* metabolic system which appears representative of mammalian metabolism. The rapid rate of metabolism indidated that metabolites would be produced in sufficient time to elicit a detectable response if indeed they were mutagenic in the Ames assay. The concentration of an active metabolite required to produce a responce is unknown and likely varies with each compound. For this reason, each chemical must be screened over a wide range of concentrations. This poses the question of the effect of concentration on the rate of metabolism. While the problem was not considered in the present study, such an investigation is planned for the future.

Another question which deserved attention concerns the effects of the microorganisms upon the metabolism of the S-9 RLH. It was demonstrated that the microbes had a negligible effect upon the parent compound but their effect upon RLH-produced metabolites has not been evaluated. The addition of excess histidine, however, did increase the concentration of 5-hydroxy carbaryl and greatly decreased the concentration of 5,6-dihydrodihydroxy naphthol after 48 hours of incubation (Table 34). The data indicate that the microorganisms may attack certain carbaryl metabolites, but late in the testing procedure. The critical time in the screening test is during the first few hours since the organisms must have sufficient time to reproduce into visable colonies subsequent to mutation.

The metabolism of carbaryl within the Ames assay system was shown to be representative of *in vivo* mammalian metabolism. The microorganisms were unable to significantly alter carbaryl and did not influence the effects of the S-9 RLH metabolism. The majority of the metabolic activity within the Ames system was attributed to the S-9 RLH. This study serves only to demonstrate that the Ames S-9 assay system is capable of metabolyzing carbaryl. However, there is no apparent reason why the system should not also metabolize other compounds of similar chemical configuration. It remains to be seen if the system metabolizes vastly different chemicals such as DDT the same as the intact organism.

Table 34. NATURE OF THE RADIOCARBON IN THE ORGANO-SOLUBLE FRACTION RESULTING FROM THE INCUBATION OF CARBARYL-1-NAPHTHYL-^{14}C WITH ALL COMPONENTS OF THE AMES SYSTEM, TREATMENT IV[a]

		% of ^{14}C in organo-soluble fraction/hr of incubation							
Metabolites resolved by TLC	R_f	0	1	3	6	12	24	48	48 + histidine[b]
Unknown 1	0.00	0.3	4.8	2.9	4.3	7.1	3.9	4.8	8.7
Unknown 2	0.04	0.0	1.9	1.8	2.5	2.5	2.3	2.3	3.4
5,6-dihydrodihydroxy carbaryl	0.14	0.0	33.3	43.7	49.7	45.1	44.2	33.5	36.9
5,6-dihydrodihydroxy naphthol	0.30	0.0	2.7	5.9	5.8	11.7	20.0	32.0	12.2
Unknown 3	0.39	0.0	3.0	5.7	7.3	5.1	5.6	3.1	5.5
N-hydroxymethyl carbaryl	0.50	0.0	10.4	8.9	4.8	3.7	3.1	2.2	3.8
4-hydroxy carbaryl	0.68	0.0	5.5	9.7	11.5	7.2	9.2	10.0	12.8
5-hydroxy carbaryl	0.79	0.0	6.9	10.6	7.7	9.6	5.5	5.5	12.3
Carbaryl	0.90	98.9	24.5	7.7	2.6	4.6	1.2	2.8	3.1
Unknown 4	0.98	0.8	7.1	3.1	1.8	3.4	5.0	3.8	1.3

[a] Petri plates containing the bottom agar, the complete top agar, the S-9 rat liver homogenate, and the mutants of the Ames system.

[b] Plates received 0.63 μg of histidine to promote microbial growth.

SYNTHESIS OF ^{14}C-CARBONYL METHYLCARBAMATES

While [^{14}C-carbonyl]-methyl isocyanate has been used widely for the preparation of radioactive insecticidal methylcarbamates, yields are often low, and in some cases reactions completely unsuccessful, because of the sensitivity of the isocyanate to moisture. This paper describes a process whereby the [^{14}C]-methyl isocyanate, in its break-seal container as received from the supplier, is reacted with the appropriate oxime or phenolic intermediate without exposing the isocyanate to atmospheric moisture. Results are presented for the insecticides carbaryl, carbofuran, aldicarb and methomyl demonstrating consistent yields of 85 to 95% using a procedure of uncommon simplicity.

Carbon-14 labeling of the carbonyl group in carbamate anticholinesterase toxicants provides an excellent tool for studying the disposition of these chemicals in various biological systems. In particular, knowledge of the metabolism of methylcarbamate insecticides has greatly increased because of the use of this and other radiolabeling techniques (Kuhr and Dorough, 1976). In addition to the accountability and ease of quantitation afforded by radiotracer techniques, ^{14}C-carbonyl labeling of the carbamates gives rapid data relative to an animal's ability to directly detoxify methylcarbamates through hydrolysis of the ester linkage, as this detoxication results in expiration of [^{14}C]-carbon dioxide (Krishna and Casida, 1966).

One widely used method of producing ^{14}C-carbonyl methylcarbamates involves esterification of the appropriate phenolic or oxime starting material with [^{14}C-carbonyl]-methyl isocyanate. However, unless extreme caution is used in handling the volatile, moisture-labile methyl isocyanate, the simple one-step reaction can be frustrating as well as expensive due to loss of the desired radiocarbon. The purpose here is to report a facile, efficient means of synthesis of aromatic and aliphatic methylcarbamates which circumvents many of the problems encountered in handling small quantities of radioactive methyl isocyanate. By this method ^{14}C-carbonyl labeling was accomplished with the insecticides carbaryl (1-naphthyl methylcarbamate), carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate), aldicarb (2-methyl-2-(methylthio)propionaldehyde O-(methylcarbamoyl)oxime, and methomyl (S-methyl-N-[(methylcarbamoyl)-oxy] thioacetimidate.

Materials and Methods

Phenolic and oxime starting materials for syntheses were obtained by alkaline hydrolysis of analytical grade methylcarbamates used in residue analysis. Subsequently, thin-layer chromatography (TLC) on silica gel plates (Silgel F-254, Brinkman Instruments, Des Plains, Ill. 60016) as detailed in Table 35 afforded purification. In this manner, 1-naphthol, carbofuran phenol (2,3-dihydro-2,2-dimethyl-7-hydroxybenzofuran), aldicarb oxime (2-methyl-2-(methylthio)propionaldehyde oxime) and methomyl oxime (S-methyl-N-hydroxy thioacetimidate) were isolated using systems A, B, C and D, respectively. [^{14}C-Carbonyl]-methyl isocyanate, 5.38 mCi/mM, was obtained from New England Nuclear, Boston, Mass. 02118. Reagent grade triethylamine was used as a catalyst. Benzene, dried over sodium, served as the solvent for the reaction.

Reactions of the various phenols or oximes with the radiolabeled methyl isocyanate were carried out in the glass break-seal vial (similar to K-892750, Kontes Glass Co., Vineland, N.J. 08360), as supplied by New England Nuclear. This vial consisted of two compartments. Compartment A contained 1.06 mg of the radioactive methyl isocyanate. Compartment B was dried with dessicated air and a twofold molar equivalent of the proper phenolic or oxime reactant was added along with 0.01 ml of triethylamine and 0.5 ml of dry benzene. In addition, a 1 cm section of 0.5 cm diameter glass rod was carefully inserted and compartment B was tightly sealed with a teflon-covered cork. Compartment A was immersed in a dry ice-acetone bath which served to condense the methyl isocyanate and create a reduced pressure within compartment A. Holding

Table 35. R_F VALUES OF STARTING MATERIALS AND PRODUCTS ISOLATED BY SILICA GEL TLC SYSTEMS.

	Solvent system			
Compound	A	B	C	D
Carbaryl	.58	.46	.58	.74
Naphthol	.84	.69	.81	.86
Carbofuran	.57	.41	.58	.71
Carbofuran phenol	.88	.65	.84	.83
Aldicarb	.36	.24	.38	.61
Aldicarb oxime	.90	.65	.84	.89
Methomyl	.11	.13	.14	.36
Methomyl oxime	.57	.34	.55	.72

A - 4:1 diethyl ether - hexane

B - 7:3 benzene - diethyl ether

C - 9:1 diethyl ether - hexane

D - 9:1 diethyl ether - acetone

the tube horizontally so that the tip of the break-seal was immersed in the benzene solution, the seal was easily broken by the glass rod upon a sudden movement of the vial. Due to the negative pressure, the benzene solution containing the nonradioactive reactant and the catalyst was rapidly withdrawn into compartment A. When this transfer was essentially complete, the reaction vial was placed in a water bath at 45°C for about 18 hours. The tube was then opened and the ^{14}C-carbonyl methylcarbamate isolated by silica gel TLC using the solvent systems previously described. Radioactive products were located on the plates by radioautogrpahy. These were quantitated by direct counting of the silica gel in 3a70B scintillation cocktail (Research Products International Corp., Elk Grove Village, IL. 60007) by means of a Packard Model 3380 liquid scintillation spectrometer.

Mass spectra of the purified synthetic products were obtained using a Finnigan Model 1015-C quadrupole instrument. Samples were introduced via the solid probe inlet and were ionized with a 70 eV electron beam.

Results and Discussion

The radiopurity of the crude reaction mixtures of synthesized methylcarbamates were 94 and 92% for carbaryl and carbofuran, and 88 and 86% for aldicarb and methomyl, respectively. The reproducibility of this synthetic method was demonstrated by yields of carbaryl ranging from 93.9 to 94.6% radiopurity in 4 separate attempts. Radioactive impurities occurred as 3 distinct products with only slight TLC molibity (R_f <.10) in the indicated solvent systems.

In addition to thin layer chromatography with authentic standards, mass spectra of the synthetic products confirmed their authenticity. Major fragment ions in these spectra are indicated in Table 36. The spectra of all compounds indicated ions of m/e 58, corresponding to CH_3NHCO^+ which originated from the methylcarbamyl moiety. With the exception of aldicarb, the spectrum of each compound exhibited molecular and phenol-

Table 36. MAJOR IONS IN THE MASS SPECTRA OF THE SYNTHESIZED METHYLCARBAMATES. PERCENT ABUNDANCES ARE LISTED IN PARENTHESES.

Compound	Mass spectra
Carbaryl	*201(3), 144(100), 127(3), 116(33), 115(43), 89(6), 63(6), 58(5) and 57(5).
Carbofuran	*221(6), 164(100), 149(74), 131(18), 123(19), 122(21), 121(13), 91(12), 77(12), 58(11) and 57(10).
Aldicarb	144(47), 100(30), 89(21), 87(50), 86(100), 85(63), 76(37), 58(62), 55(32) and 41(90).
Methomyl	*162(1), 105(76), 88(29), 58(100), 47(30), 44(29), 42(37), and 41(33).

* Molecular ions.

ic or oxime ions. In contrast, the thiomethylene group of aldicarb is labile apparently in preference to molecular ionization or decarbamylation.

All spectra were identical, except for the minor [^{14}C] contribution, to reference spectra of authentic compounds taken in this laboratory. In addition, the spectrum of carbaryl coincided with that previously published (Damico, 1965). The mass spectrum of aldicarb differed in base peak (and consequently in abundance of other ions) from spectra reported elsewhere (Damico et al., 1969). Such variance was judged to be due to differences in ionization or mass separation techniques.

In conclusion, the above method offers an uncommonly simple means of obtaining radiolabeled methylcarbamates. This method should be of wide utility due to the extensive use of methylcarbamate compounds as drugs and pesticides. Moreover, the potential for synthesis of small quantities of these materials at low cost should extend the availability of this radiolabel to any interested investigator.

THE INFLUENCE OF DOSE ON THE DETOXICATION AND ELIMINATION OF CARBAMATES IN RATS

Studies of methylcarbamate insecticide metabolism typically deal with 2 general types of chemical reactions *in vivo*, oxidation and hydrolysis. The latter process deserved more attention than it has received to date since it provides a single-step direct detoxication mechanism, is observed extensively in both animals and plants, occurs rapidly, and begins almost immediately within an organism after exposure. This report represents an attempt to focus on hydrolysis of the anticholinesterase insecticide Croneton (2-ethylthiomethylphenyl methylcarbamate) in the rat in order to examine parameters governing the efficacy of this form of detoxication under differing conditions of animal exposure.

One such parameter to be considered in any situation of animal-toxicant interaction is the level of exposure. Since hydrolysis of ^{14}C-carbonyl labeled methylcarbamate insecticides is known to yield $^{14}CO_2$ (Kuhr and Dorough 1976), it was felt that changes in hydrolysis could be examined by monitoring the expired air of rats treated with [^{14}C-carbonyl]-Croneton. In addition, excretion from rats of such radiocarbon by urinary and respiratory routes is essentially quantitative within 3 days (Nye *et al.* 1976). This knowledge, combined with the observation that nearly 90% of radioactivity from ^{14}C-labeled sodium carbonate given i.p. appeared in exhaled air within one hour of administration (Krishna and Casida 1966), suggested that the kinetics of hydrolysis could be adequately inferred from kinetics of $^{14}CO_2$ expiration following [^{14}C-carbonyl]-Croneton dosing. This is further suggested by the finding that less than 1% of urinary radioactivity excreted from rats treated with [^{14}C-carbonyl]-Croneton sulfoxide was released upon acidification of urine with 1 N HCl (Hurst and Dorough 1976), as urinary [^{14}C]-bicarbonate would be. With this implicit assumption, an examination has been undertaken of the elimination and detoxication of various doses of Croneton ranging from small quantities causing no overt toxicity to those resulting in severe poisoning.

Materials and Methods

Chemicals - Stock [^{14}C-carbonyl]-Croneton (5.88 mCi/mM), synthesized by esterification of 2-ethylthiomethylphenol with [^{14}C-carbonyl]-methyl isocyanate, was used in preparation of animal doses. This material was diluted with nonradioactive Croneton (Chemagro Division of Mobay Chemical Corporation, Kansas City, MO.) to specific activities of 2.2, 0.44, 0.044, and 0.0044 mCi/mM for doses of 100, 20, 2.0, and 0.2 mg/kg animal weight, respectively. These doses were prepared on the day of treatment by dissolving appropriate quantities in Tween-80 (Fisher Scientific Company, Fair Lawn, N.J.) such that each dose would contain about 10^6 dpm in a preset volume of vehicle (0.5 ml/250 g rat).

Animals and Treatment - All rats used were female Cox-S. D. albinos (Laboratory Supply Company, Indianapolis, IN.) weighing about 250 g each. All animals were starved overnight prior to weighing and dosing with a feeding needle. Atropinized rats were injected i.p. with 50 mg/kg atropine sulfate in 0.2 ml distilled water immediately before oral treatment. Immediately following gavage, animals were placed in glass metabolism cages (Delmar Scientific Glass Products of Coleman Instruments, Maywood, IL.) designed for separate collection of urine, feces, and respiratory gases.

Analysis - Following treatment, all animals were observed periodically and evident symptoms of poisoning were noted.

Air from each metabolism cage was withdrawn by vacuum pump through 2 CO_2 traps connected in series containing 1 N KOH. Airflow was maintained at 400 ml by periodic adjustment of a needle-valve manifold. Pairs of 20 ml traps for each cage were changed at 2 hr intervals during the initial 24 hr post-treatment, after which 200 ml traps were substituted for the 2 subsequent 24 hr intervals. Replicate 2 ml aliquots were taken from each trap, diluted with 2 ml of distilled water, and counted using 15 ml of 3a70B liquid scintillation cocktail (Research Products International Corporation, Elk Grove Village, IL.) in a Packard Model 3380/ 544 counter.

Urine was collected at intervals of 12, 18, 24, 48, and 72 hr after treatment. Each sample was assayed by direct counting and was partitioned using a slight modification of the procedure of Dorough (1971). In this, urine aliquots were diluted with saturated sodium chloride solution and then extracted with acetonitrile and chloroform. Organic and aqueous phases were then radioassayed by liquid scintillation for phase distribution of radiocarbon.

Feces were allowed to accumulate throughout the 72 hr experimental period. These were weighed and representative aliquots were air-dried overnight prior to combustion in a Packard Model 306 sample oxidizer. Combusted samples were then radioassayed as above.

Transformation of $^{14}CO_2$ Data - All data from radioassays were calculated and tabulated in terms of percent of the dose administered to each animal. In addition, the kinetics of Croneton hydrolysis as determined by $^{14}CO_2$ evolution were examined by conventional methods involving transformation of exponential funcitons into their linear equivalents (Riggs 1963). For this, the total $^{14}CO_2$ expiration occurring in the 3-day period was obtained. Then the percent of the 3-day total trapped in each successive interval throughout the first 24 hr was calculated. These data, as cumulative total excretion with time, were subtracted from 100% at each interval yielding a decreasing exponential function representing the percentage of 3-day hydrolyzed material remaining in the animal body with time. Plots of the natural logarithm of this decreasing percentage were biphasically linear with time. Regression lines were fitted to each phase by the method of least squares. Excellent line fits (typically r^2=.98 or better) were obtained in this manner. Half-times ($t_{1/2}$) for the initial (α) phase, which typically lasted for about 12 hr after dosing, and the later (β) phase were calculated from the line slopes. Calculations were performed for individual rats and are expressed as mean ± standard error values at each dosage level for comparison of variation due to dose effects with that due to individual differences.

Results and Discussion

Symptomatology evident after the experimental poisonings with doses ranging from 0.2 to 100 mg/kg is listed in Table 37. The visible consequences of administration of 0.2 and 2.0 mg of Croneton per kg rat weight can be seen to be slight or unapparent. With increasing dose, toxicity was moderate at 20 mg/kg ranging to severe at 100 mg/kg. The time of functional recovery, according to the subjective interpretation of this observer, was also considerably lengthened at the high dose. In each case symptoms were typical of those seen in situations of animal poisoning with anticholinesterase agents.

With increased dose, the relative rate of hydrolysis in terms of the quantity administered (relative hydrolysis) was observed to decrease during the first 12 hr. (It should be made clear at this point that the absolute or molar rates were obviously increased with increased dosage. However this discussion centers on rates relative to the quantity to be detoxified in order to examine the animals effective ability to maintain or return to homeostasis.) This decrease in relative hydrolysis may be examined in its different forms according to the method utilized in presentation of the data. Table 38, in which is tabulated the mean proportion hydrolyzed per time interval at the various doses, indicates a decrease in the percentage of dose undergoing hydrolysis in a given time with increased quantities. In addition, relative peak rates occur later with increased administration. These effects combine to produce a flattened $^{14}CO_2$ cumulative elimination curve at higher doses when the data tabulated in Table 39 are plotted with time. This flattenting of elimination in terms of dose occurs to the extent that hydrolysis proceeds almost linearly (about 1.5 μM/m) between 4 and 16 hr after treatment in animals receiving 100 mg/kg. Thus, peak quantities of approximately 3.1×10^{-6} moles at hour 10, 9.6×10^{-7} moles at hour 8, 2.1 x

Table 37. RELATIVE SYMPTOMS APPARENT IN RATS GIVEN VARIOUS DOSES OF [^{14}C-CARBONYL]-CRONETON

Dose (mg/kg)	Symptoms
100	Extensive tremors, ataxia, and prostration for several hours; excessive salivation to the extent that the entire underside of the animal is wet; eyes wide and bulging, exuding reddish tears several hours after dosing; death in 1 of 4 animals dosed, those surviving recovered relatively normal feeding and grooming habits by 12 hours after dosing.
20	Moderate tremors and ataxia; little or slight salivation to the extent that the chin was wet, ceasing within 3 hours; recovery of normal mannerisms 3-6 hours after dosing.
2.0	Very slight tremors; no overt excessive salivation; otherwise asymptomatic; minimal disturbance in behavior patterns.
0.2	No apparent symptoms; animals maintain normal exploratory, feeding, and grooming habits throughout interval.

Table 38. MEAN ELIMINATION FROM RATS OF $^{14}CO_2$ PER TIME INTERVAL AS A FUNCTION OF DOSE OF [^{14}C-CARBONYL]-CRONETON

	Percent of dose eliminated per interval as $^{14}CO_2$[a]			
Hour[b]	0.2 mg/kg	2 mg/kg	20 mg/kg	100 mg/kg
2	7.5 ± 1.0	5.9 ± 0.7	1.4 ± 0.3	0.6 ± 0.07
4	13.7 ± 1.4	9.3 ± 0.5	3.3 ± 0.7	1.8 ± 0.1
6	10.8 ± 1.6	7.2 ± 0.3	3.9 ± 0.8	2.3 ± 0.2
8	6.2 ± 0.4	5.7 ± 0.7	4.3 ± 0.5	2.3 ± 0.3
10	3.9 ± 0.08	2.6 ± 0.4	3.4 ± 0.4	2.8 ± 0.7
12	2.0 ± 0.06	2.1 ± 0.3	2.2 ± 0.1	2.6 ± 0.5
14	1.2 ± 0.09	1.1 ± 0.2	1.4 ± 0.3	2.6 ± 0.5
16	0.8 ± 0.03	0.7 ± 0.1	0.9 ± 0.2	2.3 ± 0.6
18	0.6 ± 0.06	0.6 ± 0.2	0.7 ± 0.2	2.0 ± 0.4
20	0.5 ± 0.09	0.4 ± 0.2	0.5 ± 0.2	1.6 ± 0.2
22	0.4 ± 0.0	0.4 ± 0.2	0.5 ± 0.2	1.2 ± 0.03
24	0.4 ± 0.0	0.3 ± 0.1	0.5 ± 0.2	1.0 ± 0.03
48	2.9 ± 0.3	2.1 ± 1.1	3.2 ± 1.0	4.4 ± 0.6
72	1.2 ± 0.3	0.9 ± 0.4	1.5 ± 0.4	1.9 ± 0.3

[a] Mean values ± standard error for 3 rats per dose.

[b] Time between dosing and end of 2 or 24 hour interval.

Table 39. MEAN CUMULATIVE ELIMINATION OF $^{14}CO_2$ FROM RATS ADMINISTERED VARYING DOSES OF [^{14}C-CARBONYL]-CRONETON

	Cumulative percent of dose as $^{14}CO_2$[a] per dose			
Hour[b]	0.2 mg/kg	2 mg/kg	20 mg/kg	100 mg/kg
2	7.5 ± 1.0	5.9 ± 0.7	1.4 ± 0.3	0.6 ± 0.05
4	21.2 ± 2.4	15.2 ± 1.1	4.7 ± 1.0	2.4 ± 0.2
6	32.0 ± 4.0	22.4 ± 1.3	8.6 ± 1.8	4.7 ± 0.3
8	38.2 ± 4.4	28.2 ± 1.0	12.9 ± 2.3	7.0 ± 0.4
10	42.0 ± 4.4	30.8 ± 1.1	16.3 ± 2.6	9.5 ± 0.6
12	44.1 ± 4.4	32.9 ± 1.1	18.5 ± 2.5	12.0 ± 0.9
14	45.3 ± 4.3	34.1 ± 1.2	20.0 ± 2.4	14.5 ± 1.4
16	46.1 ± 4.3	34.8 ± 1.3	21.0 ± 2.2	16.9 ± 1.9
18	46.7 ± 4.2	35.4 ± 1.4	21.6 ± 2.2	18.8 ± 2.3
20	47.2 ± 4.2	35.8 ± 1.5	22.1 ± 2.1	20.5 ± 2.5
22	47.5 ± 4.2	36.2 ± 1.6	22.7 ± 2.2	21.9 ± 2.5
24	47.9 ± 4.1	36.5 ± 1.7	23.2 ± 2.3	22.8 ± 2.5
48	50.8 ± 3.9	38.6 ± 2.7	26.3 ± 2.6	27.1 ± 2.3
72	52.0 ± 3.8	39.7 ± 3.2	27.8 ± 2.8	29.0 ± 2.4

[a] Mean values ± standard error for n=3 rats per dosage level.

[b] Time after single oral dose.

10^{-7} moles at hour 4, and 3.0×10^{-8} moles at hour 4 were hydrolyzed per 2 hr interval by animals receiving 100, 20, 2 or 0.2 mg/kg. These data suggest some degree of saturation at high doses of the mechanisms responsible for hydrolysis and respiratory elimination.

Further evidence of the effect of increased dose on relative hydrolysis can be observed with the increase in the mean α phase half-time of hydrolysis as shown in Table 40. These half-times of occurring hydrolysis range from about 4 hours with doses of 0.2 and 2 mg/kg to an extreme of 14.5 hr with doses of 100 mg/kg. An analysis of variance performed with data from the 12 nonatropinized rats indicated a highly significant effect ($p<.001$) due to dose. Further comparisons using the least significant difference (L.S.D.) test among the dose means (Sokal and Rohlf 1969) indicated no statistical difference at $p=.05$ level for doses of 0.2, 2, and 20 mg/kg. In contrast, the mean half-time of occurring hydrolysis in animals dosed with 100 mg/kg differed to a highly significant degree ($p<.001$) when compared with mean half-times occurring after lower doses. No influence on these $t_{1/2}$ values could be observed following pretreatment of rats with atropine.

The increase in half-time of the α-phase of occurring hydrolysis correlates well with the appearance and time course of overt symptoms of poisoning. At doses of 0.2 and 2 mg/kg, α-phase half-times are very similar (about 4 hr) and symptoms are minor or unapparent. With an increase of the dose to 20 mg/kg, this mean half time exhibits an apparent (though not statistically significant) increase and cholinergic symptoms become more prominent. The symptoms and half-times of the individual rats within the 20 mg/kg dosage and provides further correlation. Two of these rats, from which individual α-phase half-times of 4.3 and 6.0 hr were calculated, exhibited tremors and ataxis, but did not salivate excessively. The third animal dosed at 20 mg/kg was ataxic, exhibited tremors, and had salivated excessively to the point that the fur around the face was wet. These symptoms continued for about 3 hr after dosing. The α-phase of hydrolysis half-time was calculated to be 9.3 hr. At the

Table 4C. HALF TIMES OF HYDROLYSIS ($^{14}CO_2$ EXCRETION) FROM RATS ADMINISTERED VARYING ORAL DOSES OF [^{14}C-CARBONYL]-CRONETON[a]

	$t_{1/2}$ ± S.E. (hr)	
Dose (mg/kg)	α phase[c]	β phase
0.2	3.7 ± 0.3	12.8 ± 1.0
0.2 A[b]	4.0	9.5
2.0	4.0 ± 0.5	10.9 ± 2.2
20	6.5 ± 1.5	14.0 ± 0.6
100	14.5 ± 0.7	8.5 ± 1.4
100 A	14.2	11.0

[a] Values expressed are the mean ± standard error calculated from 3 rats per group.

[b] Doses labeled A represent single animals receiving in addition to the dose 50 mg/kg atropine sulfate i.p.

[c] Approximate duration of 12 hr following dose.

100 mg/kg level, the animals exhibited much more pronounced symptoms (Table 37) and the calculated mean half-time was 14.5 hr. Symptoms persisted considerably longer, also. Thus, the α-phase of hydrolysis $t_{1/2}$ may be a measurable correlate of the intensity and duration of poisoning by methylcarbamate anticholinesterases. Certainly, knowing the time course of such poisoning in which recovery or death occurs within the first few hours, the α-phase duration (0-12 hr post treatment) coincides temporally while the β-phase (14-24 hr post treatment) occurs later. Too, little or no dose correlation can be seen with the latter phase $t_{1/2}$ (Table 40) which fluctuates.

Recovery of the dose during the 3-day study was excellent at levels of 0.2, 2, and 20 mg/kg as indicated in Table 41. Urinary and respiratory elimination of the radiocarbon accounted for greater than 90% of the administered doses. At the 100 mg/kg level, however, recovery was consistently poorer and averaged about 62% of the dose. Examination of the percentage of dose collected during the period 48-72 hr after dosing revealed that substantial elimination was still occurring.

Significantly less respiratory elimination (and thus hydrolysis) occurs as the dose is increased from 0.2 to 20 mg/kg (L.S.D. tests between means, $p<.05$). Increasing the dose to 100 mg/kg did not significantly alter the proportion of the dose hydrolyzed. This trend of decreasing proportion hydrolyzed with increasing dose is manifest during the first 24 hr of the experiment. During this time, differences in the percent of dose excreted in urine are apparent also. Urinary excretion increases with the dose from 0.2 to 2 mg/kg. This might be interpreted as urinary compensation for overload of the respiratory route. With further increases in the dose to 20 mg/kg, urinary radiocarbon is maintained at about 70% of the dose while respiratory elimination declines. At the high dose, however, the percentage of urinary excretion has been reduced drastically while the respiratory proportion is maintained at about 25%, and substantially reduced total

Table 41. ELIMINATION OF RADIOCARBON FROM RATS ADMINISTERED VARYING DOSES OF ^{14}C-CARBONYL-CRONETON

Excretory route and hr after treatment	Cumulative % of dose[a]/mg/kg dose			
	0.2	2.0	20	100
C-Carbon dioxide				
6	32.0	22.4	8.6	4.7
12	43.9	32.9	18.5	12.0
18	46.6	35.4	21.6	18.9
24	47.9	36.5	23.2	22.8
48	49.9	38.6	26.3	27.1
72	52.0	39.5	27.8	29.0
Urine				
12	36.9	50.8	56.9	6.1
18	41.8	62.5	63.3	14.3
24	44.8	65.2	60.7	17.4
48	47.3	66.9	63.3	23.2
72	48.0	68.1	67.1	26.0
Feces				
72	4.9	1.8	6.9	6.5
Total				
72	104.9	109.4	101.8	61.5

[a] Values represent mean of results obtained from 3 animals per dose.

recovery occurs.

Comparison of the total ^{14}C excretion of atropinized rats (Table 42) includes a slightly slowed urinary elimination in the rat given 0.2 mg/kg. This was apparently due to decreased urine flow from the animal. In contrast, the atropinized rat given 100 mg/kg exhibited substantially increased urine flow (>20 ml/24 hr compared to 3-15 ml/24 hr in rats treated with 100 mg/kg Croneton only). This apparently enabled greater urinary excretion of radiocarbon (44% vs a mean of 26% in 72 hr) and a substantially more complete recovery of the radioactivity. These observations raise questions concerning the interaction *in vivo* of varying quantities of anticholinesterase and anticholinergic compounds on urine formation, and the possibility of using diuretic agents to promote excretion of a methylcarbamate such as Croneton which is known to form a relatively water soluble toxic metabolite (Croneton sulfoxide).

From the partitioning characteristics of the urinary radiocarbon (Table 43), information is available regarding the time course of the metabolic processes responsible for production of water soluble metabolites. In the case of Croneton, the formation of hydrophilic metabolites is apparently slow relative to hydrolysis as indicated by the small proportion of the early ^{14}C excretion which is water soluble. This water soluble fraction of excretion per interval appears to increase with time. However if the decreased percentage of the dose excreted in each succeeding interval is considered, it can be seen that organosoluble materials decline dramatically while excretion of hydrophilic material is more nearly constant.

As a brief recall of the salient trends discussed, hydrolysis expressed as a function of the dose of Croneton is slowed and diminished with increasing dosage. The degree of slowing is quite pronounced at high doses and is reflected in the increased half-time of $^{14}CO_2$ elimination. Such increases in half-time correlate well with observed toxicity as manifest by excessive cholinergic stimulation. (Salivation serves as a

Table 42. CUMULATIVE ELIMINATION OF RADIOCARBON FROM RATS ADMINISTERED [^{14}C-CARBONYL]-CRONETON AFTER RECEIVING 50 MG/KG ATROPINE SULFATE I.P.

		Cumulative percent of dose excreted per animal			
Dose (mg/kg)	Hour	CO_2	Urine	Feces	Total
0.2	6	28.6	-	-	28.6
	12	42.9	1.2	-	44.1
	18	47.2	35.4	-	82.6
	24	48.7	36.5	-	85.2
	48	51.7	39.1	-	90.8
	72	42.9	40.0	7.3	100.2
100	6	5.5	-	-	5.5
	12	12.5	23.2	-	35.7
	18	18.5	31.7	-	50.2
	24	21.6	34.7	-	56.3
	48	27.3	42.2	-	69.5
	72	29.6	44.0	9.9	83.5

Table 43. PARTITIONING CHARACTERISTICS OF URINARY RADIOCARBON FROM RATS AS A FUNCTION OF TIME AND DOSE OF [^{14}C-CARBONYL]-CRONETON

		Percent distribution between phases[a]			
Hour[b]	Phase[c]	0.2 mg/kg	2.0 mg/kg	20 mg/kg	100 mg/kg
12	O	87.4 ± 2.2	88.0 ± 3.0	79.2 ± 8.6	67.7 ± 12.7
	W	12.6	12.0	20.8	32.3
	(%)[d]	36.9 ± 1.1	38.2 ± 13.2	47.0 ± 9.7	6.1 ± 3.1
18	O	68.0 ± 4.5	62.4 ± 14.3	61.5 ± 5.0	32.2 ± 4.3
	W	32.0	37.6	38.5	67.8
	(%)	4.9 ± 0.4	11.7 ± 3.5	3.4 ± 0.3	8.2 ± 2.0
24	O	49.4 ± 11.6	37.9 ± 9.6	45.4 ± 11.5	14.6 ± 1.0
	W	50.6	62.1	54.6	85.4
	(%)	2.9 ± 1.3	2.7 ± 1.4	4.1 ± 2.6	3.1 ± 0.8
48	O	22.6 ± 6.9	13.7 ± 2.1	20.8 ± 3.0	9.4 ± 2.9
	W	77.4	86.3	79.2	90.6
	(%)	2.6 ± 0.4	1.7 ± 0.3	2.9 ± 1.4	5.9 ± 1.2
72	O	10.3 ± 1.4	11.1 ± 3.5	12.2 ± 4.8	8.8 ± 3.8
	W	89.7	88.9	87.8	91.2
	(%)	0.7 ± 0.06	1.2 ± 0.5	0.9 ± 0.2	2.8 ± 0.7

[a] Mean ± standard error for 3 rats per dosage level.

[b] Time of urine collection after single oral dose.

[c] W - saline, O - acetonitrile-chloroform phase.

[d] Mean ± standard error (n=3) percent of dose represented in total sample.

good indicator.) Diminished relative hydrolysis of Croneton with increased doses below levels producing apparent poisoning symptoms (0.2-2 mg/kg) appears to result in increased (compensatory) urinary elimination. With further increases in the dose to levels producing overt toxicity (20-100 mg/kg), relative hydrolysis diminishes to an apparent asymptote of about 25% of the dose and urinary compensation becomes increasingly less effective. Consequently at high doses, 3-day recovery of the administered radioactivity ceases to be quantitative. Administration of moderate doses of atropine sulfate does not alter hydrolysis, but may affect urinary excretion by possible influences on urine flow.

FATE OF 1-NAPHTHYL N-HYDROXYMETHYLCARBAMATE IN RATS

A major mode of metabolism of foreign substances in mammals is oxidation. In the case of aryl methylcarbamates, both N-methyl and nuclear hydroxylation are known to occur. Many studies have indicated that N-hydroxymethyl carbaryl is an important metabolite in the metabolism of carbaryl in both plants and animals (Kuhr and Dorough, 1976). For this reason, the metabolism of N-hydroxymethylcarbaryl in rats was examined in order to estimate the fate of this material when ingested as residues in the diet of man.

Materials and Methods

Chemicals - 1-Naphthyl-1-^{14}C carbaryl (15.2 mCi/mMol), carbonyl-^{14}C carbaryl (5.38 mCi/mMol), and metabolites, 5,6-dihydro-5,6-dihydroxy carbaryl, N-hydroxymethylcarbaryl, desmethyl carbaryl, and 1-naphthol were obtained from stocks maintained in our laboratory.

Chromatography - The purity of the ^{14}C-labeled and authentic compounds was determined by thin-layer chromatography. The ether extractable metabolites were separated on tlc (Brinkman precoated silica gel 60-F-0254, 250 x 250 mm^2) using the following solvent systems: A - Ether-hexane, 4:1 (v/v); B - Ether-hexane, 9:1 (v/v); C - Chloroform-acetonitrile, 2:1 (v/v).

Biosynthesis of 1-naphthyl-1-^{14}C N-hydroxymethylcarbaryl and carbonyl-^{14}C N-hydroxymethylcarbaryl were obtained from in vitro metabolism of ^{14}C-ring and carbonyl-^{14}C labeled carbaryl by rat liver microsomes. A ten gram sample of fresh rat liver was obtained from mature male albino rats (Cox-SD, Laboratory Supply Company) weighing approximately 200 g each. The liver was homogenized with a Teflon pestle in a glass homogenizer containing 40 ml of 0.1 M phosphate buffer solution (pH 7.4). The homogenate was centrifuged at 15,000g for 15 min and the 15,000g supernatant fraction centrifuged at 105,000g for 1 hr to obtain the microsomal fraction.

In in vitro experiments 1-naphthyl-^{14}C carbaryl or carbonyl-^{14}C carbaryl was deposited on the surface of the reaction vessel by evaporation of a benzene solution. Then, 3.0 ml of the microsomal fraction were added and mixture shaken for 30 min at 37°C with NADPH (1.115 mg), magnesium chloride (6.41 mg), methyl nicotinamide (6.34 mg), glucose-6-phosphate (3.92 mg), glucose-6-phosphate dehydrogenase (2 units), and glutathione (1 mg) in a total volume of 4.0 ml. Control incubations were identical except that the liver enzymes were initially heated for 30 min at 90°C.

Analysis of incubation mixture - Sodium chloride (∿2 g) was added to each incubation mixture and extracted with ether to remove carbaryl and the remaining unconjugated materials. The ether layer was concentrated to a volume which allowed direct application to silica gel thin-layer plates. Solvent systems A and B were used to resolve the radioactive components of the ether extract.

Detection of the radioactive zones on tlc plates was accomplished by means of autoradiography. Extraction of each component from the plates was by removal of the gel, sonication in acetone and then concentrating the acetone solution. Identification of each component was based on its tlc behavior and confirmed by mass spectral analysis.

In some of the experiments, rat liver homogenates were obtained from rats which had been given intraperitoneal injections of sodium pentobarbital (40 mg/kg) daily for 4 days in order to induce high levels of drug-metabolizing enzymes.

Stability of N-hydroxymethylcarbaryl - The stability of N-hydroxymethylcarbaryl during various experimental procedures used for evaluating the metabolism of the carbaryl in animals was determined. Because many of the procedures described herein could also cause degradation, this aspect was considered using ring^{14}C-labeled N-hydroxymethylcarbaryl obtained from the in vitro study. The compound was subjected to 1 N HCl, pH 5.8 buffer and neutral conditions for 24 hr. The degree of stability was determined by the amount of degradation products revealed utilizing tlc and autoradiography.

Treatment - Albino rats (Cox-SD, Laboratory Supply Co.) weighing approximately 200 g each were used in all metabolism experiments. In the single oral dose studies, female animals were treated with ^{14}C-ring and ^{14}C-carbonyl-labeled carbaryl and N-hydroxymethylcarbaryl at a dosage level of 0.5 mg/kg. The specific activity of the compounds was adjusted to 6 mCi/mMol and delivered to the animals in 0.5 ml of 30% Tween 80 via a feeding needle. Two rats were treated so that all data collected represented the results obtained from these two rats.

Sample collection and radioassay - Following treatment, the animals were placed in metabolism cages allowing for separation of urine and feces. Those treated with the ^{14}C-carbonyl-labeled materials were placed in cages modified for collection of exhaled gases. Urine samples were radioassayed by direct scintillation counting. Feces samples were collected at 48 hr after end of treatment and radioassayed by combustion using a Packard Model 306 Sample oxidizer. The $^{14}CO_2$ in the exhaled gases of rats were collected by drawing air from the cages through 100 ml of 1.0 N potassium hydroxide. This system was tested before the experiment using ^{14}C-sodium bicarbonate and found to collect 95+% of the carbon dioxide. Samples were taken for radioassay at hourly intervals for 4 hr post treatment, then at 2 hr intervals for the next 10 hr, 3-hr intervals for the next 6 hr and at different intervals for the remainder of the 48 hr experiment. Radioassay was accomplished by direct scintillation counting of 1.0 ml of the trap solution.

Analysis of urinary radiocarbon - Urine was extracted with ether and then lyophilized to dryness and the solid residue washed with methanol repeatedly until no radioactivity was detected in the wash. This process was quantitative in the recovery of the urinary radiocarbon. The methanol wash was evaporated to dryness, the solid residue disolved in 6 ml of distilled water and then extracted thoroughly with ether.

Portions of each of the water phases were incubated with 1 N HCl at 40°C for 24 hr. The incubations were conducted in a sealed tube containing 4 ml of ether. Periodic shaking of the tubes extracted the hydrolyzed exocons (or non-polar products) into the ether layer to prevent further decomposition. The ether extracts were combined and analyzed by tlc.

Enzyme preparations also were utilized to cleave possible conjugated water-soluble metabolites. The enzymes β-glucuronidase (bacterial, powder) and arysulfatase (powder) were purchased from Sigma Ltd. (St. Louis, Mo.). β-Glucuronidase hydrolysis of the conjugates (1.0 ml) was conducted in 0.1 M phosphate buffer pH 6.2 (2 ml) containing 10 units of the enzyme. The reaction mixture was incubated for 24 hr at 37°C. Arylsulfatase hydrolysis was effected by incubation of arylsulfatase (10 units) with 1 ml of the conjugates and 0.1 M sodium acetate-acetic buffer, pH 5.2 (2 ml), for 24 hr at 37°C.

Results and Discussion

In vivo metabolism of carbaryl - Degradation studies of carbaryl using subcellular liver preparation have played a major role in determining the metabolic pathway of this carbamate in animals. The results from this investigation showed that N-hydroxymethylcarbaryl and 5,6-dihydro-5,6-dihydroxycarbaryl were the major metabolites, 27 and 14% of total, found in the incubation mixture (Table 44). Almost identical amounts of these two metabolites were found using carbonyl-^{14}C carbaryl as the substrate. The microsomal fraction from rats which received sodium pentobarbital metabolized about 15% more of the carbaryl and this was reflected primarily as increased quantities of N-hydroxymethylcarbaryl

Table 44. RADIOACTIVE COMPONENTS RESULTING FROM THE METABOLISM OF 1-NAPHTHYL-1-^{14}C CARBARYL BY RAT LIVER MICROSOMES.

Metabolite	% ^{14}C in sample Untreated[a]	 Treated[b]
Unknown origin material	4.7	5.1
5,6-Dihydro-5,6-dihydroxy 1-naphthyl N-methylcarbamate	14.4	20.1
1-Naphthyl N-hydroxymethylcarbamate	27.3	37.4
Carbaryl	35.7	20.1
Water-soluble metabolites	10.1	11.4
Total ^{14}C recovery	92.2	94.1

[a] Microsomal fraction obtained from rats which did not receive sodium pentobarbital treatments.

[b] Microsomal fraction obtained from rat treated with sodium pentobarbital.

and 5,6-dihydro-5,6-dihydroxy carbaryl.

Stability of N-hydroxymethylcarbaryl - When ring-^{14}C-labeled N-hydroxymethylcarbaryl was incubated with 1 N HCl for 24 hr at 37°C, 3 radioactive materials were produced (Table 45). Two radioactive materials were identified as desmethyl carbaryl (19%) and 1-naphthol (3.7%). The other material remained at the tlc origin and accounted for only 2% of the total radiocarbon. In acetate buffer solution (pH 5.2), N-hydroxymethylcarbaryl was more stable than in 1 N HCl when incubated under identical conditions. However, 6% desmethyl carbaryl was produced under these conditions. In water, only a small amount (1.2%) of desmethyl carbaryl was formed. In all of the stability studies, desmethyl carbaryl was the major decomposition product. This information makes it clear that desmethyl carbaryl would be produced if the hydroxymethyl metabolite was subjected to acid conditions normally used to cleave conjugated materials.

Excretion - The recovery of radioactivity from rats treated with ^{14}C-ring-labeled carbaryl and N-hydroxymethylcarbaryl (orally, 20 mg/kg) is presented in Table 46. Total ^{14}C-recovery after 48 hr from rats dosed with ^{14}C-ring-labeled carbaryl was 88%, with 82% in the urine and 6% in the feces. For ^{14}C-ring-labeled N-hydroxymethylcarbaryl, total ^{14}C recovery after 48 hr was 86%; 72% was in the urine and 13% in the feces (Table 46). The amount of elimination of urinary metabolites in the carbaryl- and N-hydroxymethylcarbaryl-treated rats was distinctly different during the first 10 hr. Approximately 38% as compared to 64% was excreted in the urine of rats dosed with carbaryl and N-hydroxymethylcarbaryl, respectively. The difference in ^{14}C excretion in the urine during the first 10 hr after treatment probably resulted from the hydroxy group on the methyl group of the metabolite. This hydroxy group would likely form conjugates very rapidly and, thereby, facilitate the excretion of N-hydroxymethyl carbaryl.

Table 45 . THE CHEMICAL STABILITY OF RING-^{14}C-N-HYDROXYMETHYLCARBARYL STORED UNDER DIFFERENT CONDITIONS FOR 24 HR AT 37°C.

Products	% of radiocarbon as indicated product 1 N HCl	 pH 5.2 acetate buffer	 pH 7.0 water
Unknown origin product	2.1	1.0	0.0
N-Hydroxymethyl carbaryl	70.6	91.3	97.7
Desmethyl carbaryl	19.4	6.0	1.2
1-Naphthol	3.7	0.0	0.0
Total	95.8	98.3	98.9

Table 46. URINE AND FECAL ELIMINATION OF RADIOCARBON BY RATS TREATED WITH ^{14}C-RING-LABELED CARBARYL OR N-HYDROXYMETHYLCARBARYL.

Substrate and hours	Cumulative % of dose[a]					
	Carbaryl			N-Hydroxymethylcarbaryl		
	A	B	Avg	C	D	Avg
Urine						
5	18.8	20.0	19.4	47.7	50.8	49.2
10	35.3	39.4	37.4	65.7	63.5	64.6
20	75.3	72.1	78.7	70.6	69.9	70.2
30	81.1	82.0	81.6	71.4	70.7	71.0
48	83.9	82.1	83.0	74.2	72.1	73.2
Feces						
48	6.4	5.7	6.0	14.2	11.2	12.7
Total						
48	90.3	87.8	89.0	88.4	83.3	85.9

[a] Animals A and B treated orally with 20 mg/kg carbaryl (1.1×10^6 dpm) while C and D were treated with N-hydroxymethylcarbaryl at the same dose (1.5×10^6 dpm).

Administration of ^{14}C-carbonyl-labeled carbaryl and N-hydroxymethylcarbaryl to rats as a single oral dose resulted in rapid elimination of the radiocarbon as carbon dioxide (Table 47). An average of 26% of the dose was exhaled as CO_2 in rats treated with ^{14}C-carbonyl-labeled carbaryl, as compared to 53% of the dose in rats treated with ^{14}C-carbonyl-labeled N-hydroxymethylcarbaryl. This difference occurred predominantly within the first 20 hr after treatment, and demonstrated that the hydroxyl group on the N-methyl portion of carbaryl increased its susceptibility to hydrolytic attack.

Elimination of urinary radiocarbon in rats dosed with carbonyl-^{14}C-labeled carbaryl and N-hydroxymethylcarbaryl was 60% and 30% of the doses, respectively (Table 48). The difference occurred mainly within the first 20 hr after treatment. This difference resulted from the comparatively more hydrolytically stable carbaryl which reduced the loss of the carbonyl carbon as carbon dioxide. When all routes of elimination were considered (Table 49), there was no difference between carbaryl and hydroxymethylcarbaryl. In each case, 90% of the orally administered doses was eliminated from the body after 48 hr.

Analysis of urine - Ether extraction of the urine removed less than 1% of the ^{14}C materials. Therefore, no further analyses were attempted on this phase. Because most of the ^{14}C-residues remained in the urine after extraction with ether, emphasis was placed on the identification of the water-soluble metabolites. Table 50 shows the results of the conversion of water-soluble materials in the urine of rats dosed with ^{14}C-ring- and ^{14}C-carbonyl-labeled N-hydroxymethylcarbaryl to ether-soluble materials by enzyme and acid hydrolysis.

β-Glucuronidase and sulfatase treatments of the polar metabolites released 25% and 4% ether-soluble materials, respectively, from the urine of rats dosed with ring- and $^{14}C{=}O$ N-hydroxymethylcarbaryl. The exocon released from the urine of rats treated with carbonyl-^{14}C N-hydroxymethylcarbaryl by glucuronidase was all N-hydroxymethyl-

Table 47. RECOVERY OF $^{14}CO_2$ FROM RATS TREATED ORALLY (20 mg/kg) WITH CARBONYL-^{14}C-CARBARYL OR CARBONYL-^{14}C-N-HYDROXYMETHYLCARBARYL.

	Cumulative % of orally administered dose[a]					
	Carbaryl			Hydroxymethylcarbaryl		
Hours	A	B	Average	C	D	Average
1	0.5	1.1	0.8	4.3	4.4	4.4
2	1.5	2.1	1.8	10.8	8.6	9.7
3	1.6	3.5	2.5	14.6	11.3	12.9
4	2.9	5.0	3.9	17.5	14.1	15.8
6	7.0	8.2	7.6	21.3	17.8	19.6
8	11.1	12.1	11.6	25.8	22.5	24.1
10	13.8	14.6	14.2	29.5	27.6	28.6
12	17.2	17.8	17.5	36.5	37.0	36.7
14	19.4	19.6	19.5	40.0	41.7	40.8
17	21.8	21.7	21.8	47.1	45.5	46.3
19	22.5	22.6	22.5	49.0	50.0	49.0
22	23.9	23.7	23.8	51.0	53.5	52.2
26	25.0	24.5	24.7	51.1	53.6	52.3
32	25.6	25.0	25.3	51.7	53.9	52.8
48	26.4	25.6	26.0	51.9	54.1	53.0

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[a] A and B rats were dosed with carbonyl-^{14}C carbaryl (2,375,250 dpm each), while C and D rats received carbonyl-^{14}C N-hydroxymethylcarbaryl (1,253,040 dpm each).

Table 48. EXCRETION OF ^{14}C IN THE URINE OF RATS TREATED ORALLY (20 MG/KG) WITH CARBONYL-^{14}C CARBARYL OR CARBONYL-^{14}C N-HYDROXYMETHYLCARBARYL.

	Cumulative % of orally administered dose of rat[a]					
	Carbaryl			Hydroxymethylcarbaryl		
Hours	A	B	Average	C	D	Average
5	15.0	15.2	15.1	6.8	5.8	6.3
10	27.3	24.5	25.9	13.2	10.9	12.1
14	38.2	36.2	37.2	16.5	18.5	17.5
19	47.9	45.8	46.8	20.2	26.2	23.2
30	60.1	56.3	58.2	26.4	29.6	28.0
48	61.2	58.4	59.8	28.8	31.2	30.0

Table 49. ELIMINATION OF RADIOACTIVITY FROM RATS TREATED ORALLY (20 MG/KG) WITH CARBONYL-^{14}C CARBARYL OR CARBONYL-^{14}C N-HYDROXYMETHYLCARBARYL.

	Cumulative % of orally administered dose after 48 hr			
Animal[a]	$^{14}CO_2$	Urine	Feces	Total
A	26.4	59.9	4.3	90.6
B	25.6	59.7	3.9	89.2
C	51.9	28.8	7.6	88.3
D	54.1	31.2	6.5	91.8

[a] A and B rats dosed with carbonyl-^{14}C carbaryl (2,375,250 dpm each), while C and D rats received carbonyl-^{14}C N-hydroxymethylcarbaryl (1,253,040 dpm each).

Table 50 . ATTEMPTED CLEAVAGE OF WATER SOLUBLE METABOLITES IN URINE OF RATS TREATED ORALLY WITH ^{14}C-RING- OR ^{14}C-CARBONYL-LABELED N-HYDROXYMETHYLCARBARYL

	% distribution after treatment/label					
	^{14}C-Ring			^{14}C-Carbonyl		
Treatment	Ether	Water	Total	Ether	Water	Total
Glucuronidase	25.1	73.2	98.3	8.6	90.2	98.8
Sulfatase	4.1	94.5	98.6	1.1	97.0	98.1
0.5 N HCl	63.5	31.8	95.3	20.0	61.9	81.9
1.0 N HCl	74.2	19.1	93.3	30.4	51.9	82.3

carbaryl.

Acid hydrolysis of the urinary metabolites from rats dosed with ring-^{14}C hydroxymethylcarbaryl yielded exocons which were recognized as 1-naphthol (97%), and a small amount of N-hydroxymethylcarbaryl (2%) and desmethyl carbaryl (1%). Very small quantities, about 5%, of N-hydroxymethyl and desmethylcarbaryl were detected in the ether fraction after acid hydrolysis of urine from rats treated with carbonyl-^{14}C N-hydroxymethylcarbaryl. The remainder of the radioactivity in the ether, up to 25% of the total ^{14}C in the sample, was lost during evaporation and/or tlc analysis. Since this loss was not apparent with the ring-labeled ether solubles, it is evident that the volatile and/or labile carbonyl-labeled product did not contain the ring moiety. These data suggest that the N-hydroxymethylcarbamate fragment of the molecule formed upon hydrolysis does not degrade entirely to carbon dioxide as does the carbamate group from carbaryl, but to a compound having sufficient stability and polarity for urinary elimination.

BIOAVAILABILITY OF BOUND AND WATER-SOLUBLE CARBAMATE INSECTICIDE RESIDUES

The presence of chemical residue in food and nonfood crops resulting from the commercial use of pesticides is well documented (Pimentel, 1971). Most residue analysis methods utilize apolar solvents for extraction and are designed to detect the parent compound and perhaps one or two metabolites. The polar and bound products are largely overlooked. However, countless plant metabolism studies with radiolabeled compounds have revealed many examples where a considerable portion of the plant pesticide residue burden is of a polar and bound nature (Casida and Lykken 1969, Shumabukuro *et al.* 1971, Kuhr and Dorough 1976). This is particularly applicable to such widely used chemical classes of pesticides as the carbamates, thiocarbamates, and s-triazines. Recently, more interest has been directed toward evaluating the toxicological significance of the bound and water-soluble residues, and the possibility that they might possess harmful biological activity has been recently pointed out (Dorough 1976).

In order for the bound and polar metabolites to express any toxic action, though, they must first be available for absorption by biological systems. Only a few studies exist in the literature which have examined this parameter in mammals. The water-soluble metabolites of carbaryl naphthyl-[^{14}C]-treated bean plants were shown to be bioavailable after oral administration to rats (Dorough and Wiggins, 1969). No investigation was carried out on the unextractable metabolites of carbaryl. Paulson *et al.* (1975) concluded that the water-soluble products of profam phenyl-[^{14}C] from alfalfa plants were bioavailable when fed to rats, and that the bound residue was not. The unavailability of the bound metabolites was based on 86% of the dose being eliminated via the feces in four days, however, the role of biliary excretion of metabolites into the feces was not determined. Eighty-nine percent of a dose of propanil phenyl-[^{14}C] unextractable metabolites from rice plants to rats was eliminated via the feces and 11.5% was found in the urine

(Sutherland 1976). Only .05% of a similar ^{14}C dose to biliary fistulated mice and dogs appeared in the bile, thus the bound residue of propanil was deemed to be of low toxicological concern.

A need exists for complete studies which include the role of biliary excretion in determining the bioavailability of bound and polar residues of several chemical classes of pesticides. This paper reports the results of such investigations in rats on the residues from bean plants treated with ^{14}C-labeled preparations of a series of structurally diverse carbamate insecticides.

Throughout this text the terminology defined by Dorough (1976) at the ACS sponsored symposium on bound and conjugated pesticide residues is followed. "Water soluble metabolites" (WSM) and "conjugates" are used interchangeably, as are "unextractable metabolites" (UM) and "bound residue". "Endocon" refers to that portion of a conjugate derived from an endogenous compound, and "exocon" to that portion derived from an exogenous or foreign compound. "Bioavailable" means capable of being absorbed from the gastrointestinal tract following oral administration to animals.

Materials and Methods

Chemicals - The radiochemicals used in this study (Table 51) were carbaryl ring-[^{14}C] (19.7 mCi/mM), carbofuran ring-[^{14}C] (2.85 mCi/mM), Croneton ring-[^{14}C] (7.05 mCi/mM), Croneton carbonyl-[^{14}C] (5.88 mCi/mM), and aldicarb thiomethyl-[^{14}C] (2.03 mCi/mM). Ring-[^{14}C] carbofuran was purified by streaking a Brinkman precoated thin layer chromatography (tlc) plate (silica gel 60-G-254) with the radioactive preparation and developing it in 7:3 benzene:ether with a carbofuran standard. The parent band was scraped off and extracted from the silica gel with acetone, utilizing an Artek sonic dismembrator. Aldicarb thiomethyl-[^{14}C] was purified on a microcolumn packed with Mallinckrodt SilicAR cc-7 silica gel and eluted with 3:1 ether:acetone. Radiopu-

Table 51. RADIOACTIVE INSECTICIDES USED IN THE TREATMENT OF BEAN PLANTS

Chemical identity and position of carbon-14	Designation	Sp. act. (mCi/mMol)
1-Naphthyl-[^{14}C] methylcarbamate	carbaryl ring-[^{14}C]	19.70
2,3-Dihydro-2,2-dimethyl-7-benzo-[^{14}C]-furanyl N-methylcarbamate	carbofuran ring-[^{14}C]	2.85
2-Ethylthiomethylphenyl-[^{14}C] N-methylcarbamate	Croneton ring-[^{14}C]	7.05
2-Ethylthiomethylphenyl N-methylcarbamate-carbonyl-[^{14}C]	Croneton carbonyl-[^{14}C]	5.88
2-Methyl-2-(methyl-[^{14}C]-thio) propionaldehyde O-(methylcarbamoyl) oxime	aldicarb thiomethyl-[^{14}C]	2.03

rity of aldicarb was then confirmed by tlc with a standard using 6:3:2 ether:hexane:acetone as the solvent system. Radiopurity of carbaryl ring [^{14}C], Croneton ring and Croneton carbonyl [^{14}C] was determined by tlc using the following solvent systems: carbaryl, 4:1 ether: hexane and Croneton, 6:5 acetone:hexane.

The insecticide metabolite standards used for tlc were obtained from existing stocks in our laboratory. These standards were either synthesized previously in our lab or obtained from the developer. Their purity was checked utilizing the appropriate solvent systems outlined above. β-Glucosidase, β-glucuronidase Type V-A, and sulfatase Type III were acquired from Sigma Chemical Company, and glusulase was purchased from Endo Laboratories, Inc.

Animals - Albino rats (Cox-SD variety) weighing about 200 to 400 g were obtained from the Laboratory Supply Co., Indianapolis, Indiana. They were housed in Hoeltage suspended wire cages and given Purina Laboratory Rat Chow and water *ad libitum* until used in the experimental investigations.

Radioassay - A Packard scintillation spectrometer, model 3380 equipped with a Packard 544 absolute activity analyzer, was used in quantifying radioactivity. Direct scintillation counting was accomplished using scintillation cocktail type 3a70B (Research Products International). Other samples were combusted in a Packard Model 306 sample oxidizer; the evolved $^{14}CO_2$ trapped with Carbosorb (Packard), and counted after dispensing in Permafluor 25X liquid scintillator.

Chromatography - Thin layer chromatography (tlc) was carried out using Brinkman precoated tlc plates with a 250 micron thickness of silica gel 60-F-254. All tlc was performed using the appropriate standards described in the chemicals section. Identity of carbofuran-[^{14}C] metabolites was made by developing the tlc plates two-dimensionally, first in 7:3 benzene:ether, and second in 4:1 dichloromethane:acetonitrile. Similarly, carbaryl-[^{14}C] metabolites were analyzed by tlc two dimensionally in 4:1 ether:hexane and 2:1 chloroform:acetonitrile, or single dimensionally in 4:1 ether:hexane. Aldicarb- and Croneton-[^{14}C] metabolites were analyzed single dimensionally by tlc in 3:1 ether:acetone and 6:5 acetone:hexane, respectively. Radioactive spots were located by radiography on Kodak X-ray film. Carbofuran, carbaryl, and Croneton standards were located by spraying the developed tlc plates with a 1% methanolic solution of p-nitrobenzenediazonium fluoborate to detect phenols as yellow spots. The plates were then sprayed with a 5% methanolic solution of KOH to hydrolyze the carbamate-type standards, then sprayed again with the p-nitrobenzenediazonium fluoborate to detect the resulting phenols as yellow spots. Aldicarb standards were visualized by spraying the tlc plates with a 1% aqueous solution of potassium permanganate.

Quantitation of the radioactive spots was carried out by scraping the radioactive area of tlc plates into a scintillation vial containing 2 ml of methanol, pulverizing the silica gel on a sonic dismembrator, and analyzing directly by liquid scintillation counting.

Treatment of bean plants and collection of metabolites - Bean plants, ten days old, were stem injected by puncturing a small hole with a needle at the base of the plant, then administering the 35-μl dose in a second hole approximately 8 cm above the soil level with a Hamilton 50 μl syringe. Ten plants were treated with carbaryl ring [^{14}C] (5.0×10^6 dpm per plant, 23 μg) and 10 with carbofuran ring [^{14}C] (5.0×10^6 dpm per plant, 176 μg). Twenty plants were treated with Croneton carbonyl [^{14}C] (2.5×10^6 dpm, 43 μg), 20 with Croneton ring [^{14}C] (2.5×10^6 dpm, 36 μg), and 20 with aldicarb thiomethyl [^{14}C] (2.5×10^6 dpm, 106 μg). The plants treated with any one of the insecticides are referred to as a group. Some phytotoxiciy was noted in the carbofuran group due to the acetone vehicle. All plants were maintained in a greenhouse for 20 days after treatment in a peat moss:soil mixture containing plant nutrients.

Fifteen 10-day-old bean plants were stem injected with 500 μg of carbaryl (100 μl of a 5 mg carbaryl/ml of 5:1 water:acetone) which contained a small amount of carbaryl ring [^{14}C] as a tracer. The specific activity of the preparation was .00850 mCi/mM and each plant received 46,500 dpm. Eight control plants were included in this study. All plants were maintained in an environment chamber (10 hour light cycle at a temperature of 27°C, and a 14 hour dark cycle at 18°C) for 20 days thereafter in a peat moss:soil mixture containing plant nutrients.

At 20 days post-treatment, each plant was cut at the soil level, sealed in a plastic bag, and stored in a freezer until extracted. For extraction, the bean plants treated with each insecticide were divided into three subgroups and extracted separately. This was done to determine the reproducibility of the extraction procedure. One of the subgroups from each treated group of plants was further divided into epicotyl leaves, the remaining leaves, and stems and each of these plant parts were extracted separately. This was done to determine the distribution of radioactivity within the plants.

The plant material of each subgroup or plant part was cut into small pieces and homogenized thoroughly in about 100 ml of acetone. This was then filtered, re-extracted once with acetone, filtered, and re-extracted with chloroform. The filtrates were combined in a separatory funnel with 25 ml of water, and shaken thoroughly with 300 ml of chloroform. After separation of the phases, the organic solvent layer was drained into a clean separatory funnel and washed twice with 15 ml of water. Samples of the organoextractable (containing free metabolites) and water soluble fractions (containing WSM) were removed for radioassay. The plant solids were then subjected to a Soxhlet extraction overnight (about 16 hours) with 150 ml of methanol. Prior to radioassay of the Soxhlet extract, the solvent was centrifuged for 5 minutes to remove the particulate material. The amount of unextractable [^{14}C] (UM) in the plant solids was determined by oxidizing about

100 mg of the solids in a Packard Tri-Carb 306 combuster and assaying the collected $^{14}CO_2$.

Sorghum plants were treated in the field by Chemagro Chemical Company five times at 10-day intervals. Sampling occurred 14 days after the last treatment. Ring-labeled ^{14}C-Croneton was applied with an aerosol spray apparatus as an emulsifiable liquid formulation in water with a specific activity of 3.63 mCi/mmol. The application rate was eight ounces AI/acre.

The sorghum leaves were each coarsely chopped in a Waring blender. They were then weighed and homogenized at high speed for 5 min in a Virtis 45 homogenizer containing a 100 ml mixture of 8:2 acetonitrile and water. The homogenate was filtered under vacuum using Whatman No. 2 filter paper. The solid residue was re-homogenized in the same solvent mixture using a polytron homogenizer (Brinkman) and filtered. Aliquots of both the organic and water layers were radioassayed by liquid scintillation counting. The solids were dried, combusted in a Packard Model 306 Oxidizer and radioassayed.

Treatment of rats and collection of radiocarbon - Female rats were housed one animal per cage in Delmar metabolism cages which separate urine and feces and allow for the collection of expired gases. Before administration of WSM to rats, one animal was treated with the concentrated water solubles of five untreated 20-day-old bean plants, and observed for 24 hours to determine if any lethal or sublethal toxicity from this fraction existed. The rats were acclimated to the metabolism cages and deprived of feed overnight, but given water *ad libitum*. The WSM of carbaryl ring-[^{14}C] (1.0 x 10^6 dpm, 4.6 μg), carbofuran ring-[^{14}C] (1.0 x 10^6 dpm, 35.2 μg), Croneton ring-[^{14}C] (1.0 x 10^6 dpm, 14.4 μg), Croneton carbonyl-[^{14}C] (1.1 x 10^5 dpm, 1.9 μg), and aldicarb thiomethyl-[^{14}C] (1-0 x 10^6 dpm, 42.4 μg) were concentrated by lyophilizing to dryness and administered orally via a stomach tube in an aqueous solution that did not exceed 2 ml.

The UM of carbaryl ring-[^{14}C] (1.53×10^6 dpm, 7.0 μg) and carbofuran ring-[^{14}C] (6.5×10^6 dpm, 22.9 μg) from bean plants were fed to rats and the fate of the radiocarbon examined. Four rats were deprived of feed for 18 hours. They were then acclimated to a feeding schedule of 2 grams of laboratory chow mixed with 0.5 grams of dried, non-treated bean plant solids per day. This was done for 2 days. On the third day, the respective UM were mixed into the 2 grams of feed for each rat.

$^{14}CO_2$ was trapped in a 1.0 N KOH solution which was changed every 12 hours to prevent saturation. Samples of the KOH were taken every 6 hours and assayed for radiocarbon content by direct scintillation counting. Urine was collected every 6 hours and 0.5 ml samples assayed by direct counting. Feces were collected at each 6 hour interval after treatment if they were available. Each fecal sample was weighed, emaciated, and a 0.5 gram portion combusted for radioassay.

Cannulation of bile ducts was carried out by anesthetizing the rats with ether, shaving the ventral mid-body, and making a midline abdominal incision. The common bile duct was located, and after removing the adhering tissue, a small incision was made in the duodenal portion of the duct with iris scissors. A 12 in length of sterile polyethylene tubing (.038 O.D.) fit with a 25 G 1/2 in blunt needle was introduced approximately 3 mm into the bile duct and secured by ligature. A stab incision was made in the tail approximately 1 in from the base with a 6 in 12 G stainless steel luer-lok needle fit with a stylus, which was then thrust forward subcutaneously to the midline incision. The bile duct cannula was then fed through the needle, and hence emerging from the stab incision in the tail after removal of the needle. The abdominal incision was then sutured, and the skin closed with Clay Adams 9 mm stainless steel staples. These rats were then placed in modified Hoeltoge plastic metabolism cages, which allowed for restraining of the animal by leading the tail through an enlarged hole in the feeder. The tail was then secured in this position by affixing a No.

7 stopper to the tail with Eastman 910 Adhesive and strapping adhesive tape aroung the tail just caudally to the stopper. Bile was collected on an hourly basis using an Isco model 1200-Pup fraction collector. Sham-operated animals were carried through all the surgical manipulations with the exception of cutting and ligating the bile duct. All experimental animals were restrained and housed in the same manner as the bile duct cannulated rats.

Each biliary fistulated or sham-operated rat was treated with one of the following: carbaryl ring-[^{14}C] WSM (3.2×10^5 dpm, 1.5 µg), carbofuran ring-[^{14}C] WSM (3.0×10^5 dpm, 10.6 µg), aldicarb thiomethyl-[^{14}C] WSM (5.8×10^5 dpm, 24.7 µg), Croneton ring-[^{14}C] UM from sorghum (3.0×10^5 dpm, 8.5 µg), carbaryl ring-[^{14}C] UM from bean plants (5.4×10^5 dpm, 2.6 µg), or carbofuran ring-[^{14}C] UM from bean plants (3.6×10^5 dpm, 12.7 µg).

Metabolite Analysis - The water soluble fractions from the treated bean plants were lyophilized to dryness and the solid residue washed repeatedly with methanol until no radioactivity was detected in the wash. This process was quantitative in the radiocarbon recovery of the WSM, while leaving much of the interfering plant material behind. The methanol wash was then concentrated to about 10 ml and stored in a freezer.

For enzyme treatment of the WSM, a portion of the concentrated methanol wash, amounting to 200,000 dpm, was removed and evaporated to dryness under a gentle stream of nitrogen in 15 ml centrifuge tubes. Five ml of acetate buffer (pH=5.6) was added to each tube, as was 20 units of glucosidase or 20 units of sulfatase. The tubes were then incubated for 24 hours at 37°C in a shaking water bath. The released exocons were extracted from the buffer with three equal volumes of ether (5 ml) and samples removed for radioassay. The ether extracts were then concentrated under a gentle stream of nitrogen and applied to silica gel plates for tlc analysis.

Portions of the plant solids containing the UM of the insecticides and amounting to 500,000 dpm each were removed for treatment with acid. The solids were finely pulverized with a mortar and pestle, and mixed into about 30 ml of 1.0 N HCl in 50 ml test tubes. The test tubes were then placed into a boiling water bath and the contents mixed periodically for 2 hours. The released exocons were extracted with three equal volumes of ether (30 ml) and samples taken for radioassay. The ether extracts were then concentrated under a gentle stream of nitrogen and applied to silica gel plates for tlc analysis. The solids were removed from the acid mixture by centrifugation and the aqueous portion radioassayed.

The samples of urine from each rat were pooled, extracted with 20 ml of chloroform, and lyophilized to dryness in 250 ml round bottom flasks. The solid residue was washed repeatedly with methanol until no radioactivity was detected in the wash. This process left many of the interfering urinary components behind, and where it proved quantitative in the recovery of urinary radiocarbon, the methanol was concentrated and samples removed that contained 200,000 dpm each for enzyme and acid treatments. The urine from rats treated with Croneton carbonyl [^{14}C] WSM contained only 80,000 dpm, therefore the samples for enzyme and acid treatments contained only 20,000 dpm. The methanol was evaporated under a gentle stream of nitrogen. The methanol extraction of lyophilized urine from rats treated with WSM of carbaryl ring [^{14}C] was not efficient at removing all the radiocarbon, thus the residue from this urine was washed twice with 5 ml of distilled water. The methanol wash of the carbaryl WSM urine was evaporated to dryness and the water wash was combined with this radiocarbon. Four samples were removed that contained 200,000 dpm each and added to 15 ml centrifuge tubes.

To the urinary radiocarbon samples of Croneton ring [^{14}C] WSM, Croneton carbonyl-[^{14}C] WSM, carbaryl ring-[^{14}C] WSM, carbofuran ring-[^{14}C] WSM, and aldicarb thiomethyl-[^{14}C] WSM treated rats were added 20 units of glucosidase in 5 ml acetate buffer (pH=5.6), 20 units of glucuronidase in 5 ml acetate buffer, 20 units of sulfatase in 5 ml of acetate buffer, or 10 ml of 1.0 N HCl. The samples of urinary radiocarbon from rats administered carbofuran ring-[^{14}C] UM were treated with 20 units of glucuronidase, or 20 units of sulfatase. The sample of urinary radiocarbon from rats administered carbaryl ring-[^{14}C] UM was treated with 0.5 ml of glusulase. The enzyme treated samples were incubated in 15 ml centrifuge tubes in a shaking water bath at 37°C for 24 hours. The acid treated samples were placed in a boiling water bath in 25 ml test tubes for 1 hour. The released exocons were extracted with three equal volumes of ether (5 ml) and radioassayed. The ether extracts were then concentrated under a gentle stream of nitrogen and applied to silica gel plates for tlc analysis.

The feces from rats treated with carbaryl and carbofuran UM were extracted twice with 100 ml of acetonitrile and filtered. The filtrate was partitioned between 50 ml of water and 50 ml of chloroform, then the chloroform-acetonitrile fraction was washed twice with 15 ml of water. The aqueous portions were combined and all fractions were radioassayed.

Results and Discussion

Fate of insecticides in bean plants - An investigation of the radiocarbon distribution in treated bean plants revealed that the epicotyl leaves of plants treated with carbofuran ring-[^{14}C], Croneton ring-[^{14}C], and aldicarb thiomethyl-[^{14}C] contained 70, 90, and 90% of the dose, respectively. Since the bound and water-soluble residue from these insecticides were to be administered to rats, it was desirous to obtain preparations of these metabolites that were high in specific activity with a minimum of extraneous plant material. Therefore, only the epicotyl leaves of plants treated with the three above mentioned preparations were extracted and used in this study. Likewise, 78% of the radiocarbon recovered from five plants treated with Croneton carbonyl-[^{14}C] was found in the epicotyl leaves, thus all subsequent extractions and tests involving bean plants containing equivalents of this preparation were carried out with the epicotyl leaves only. In contrast to the other insecticides studied, a sample of five carbaryl ring-[^{14}C] treated plants had a radiocarbon distribution of 21.2% of the dose in the epicotyl leaves, 17.8% in the remaining leaves, and 60.9% in the stems. Thus, the entire bean plant of all those treated with carbaryl ring-[^{14}C] was extracted and used in this study.

Table 52 illustrates the nature of the radiocarbon extracted from bean plants. The tendency for bound residue to accumulate varied markedly with a startling 50.6% of the dose for the carbaryl ring-[^{14}C] treated plants, while the amount found with the remaining insecticide applications were well below this figure at 7.0, 3.3, 1.3, and 2.2% for carbofuran ring [^{14}C], Croneton ring-[^{14}C], Croneton carbonyl [^{14}C], and aldicarb thiomethyl-[^{14}C], respectively. WSM accounted for 30.5, 56.6, 40.6, 2.6, and 29.3% of the dose for carbaryl, carbofuran, Croneton ring-[^{14}C], Croneton carbonyl-[^{14}C], and aldicarb, respectively. It is evident that the combined UM and WSM from the ring-labeled aryl carbamates was about equal to or greater than the organosoluble or free metabolites. However, the aldicarb data presented a different picture with the organosoluble metabolites amounting to almost twice the UM and WSM. These results are not unlike those seen in other studies conducted on the plant metabolism of carbamate insecticides (Dorough 1968, Dorough and Wiggins 1969, Coppedge *et al.* 1967, Bartley *et al.* 1970). The low radiocarbon recovery of carbonyl-[^{14}C] Croneton (17.8% of the dose) as compared with that of ring-[^{14}C] Croneton (89.9%) indicated hydrolysis of the carbamic acid ester in the plants and loss of the ^{14}C label as $^{14}CO_2$.

Fate of bean plant residues in rats - When the UM of carbaryl and carbofuran were administered to rats as a dietary supplement, 99.5 and 78.8% of the dose was eliminated via the feces in 48 hours, while the urinary excretion amounted to 1.3 and 8%, respectively (Table 53). In the same time span, only 0.3 and 1.4% of an identical UM dose was

Table 52. NATURE OF RADIOCARBON IN BEAN PLANTS TREATED WITH [^{14}C]-LABELED CARBAMATE INSECTICIDES[a]

Insecticide Treatment	Mean percent of total injected and standard error[b]		
	organo-soluble	water-soluble (WSM)	bound (UM)
Carbaryl ring-[^{14}C][c]	19.2 ± 1.9	30.5 ± 1.2	50.6 ± 7.3
Carbofuran ring-[^{14}C][d]	14.5 ± 0.6	56.6 ± 3.4	7.0 ± 0.3
Croneton ring-[^{14}C][d]	45.9 ± 1.3	40.7 ± 0.2	3.3 ± 1.0
Croneton carbonyl-[^{14}C][d]	13.9 ± 2.0	2.6 ± 0.2	1.3 ± 0.4
Aldicarb thiomethyl-[^{14}C][d]	57.9 ± 3.2	29.3 ± 1.1	2.2 ± 0.4

[a] Data compiled from bean plants extracted 20 days after stem injection of the respective insecticide preparation.

[b] Combined results on three groups of plants with each insecticide.

[c] Whole plant extracted due to radiocarbon distribution.

[d] Epicotyl leaves only extracted due to radiocarbon distribution.

Table 53. ELIMINATION OF RADIOCARBON FROM RATS TREATED WITH CARBARYL, CARBOFURAN, AND CRONETON UNEXTRACTABLE METABOLITES FROM PLANTS

Unextractable metabolites	Hours after treatment	Cumulative % of dose: Intact rat[c] Urine	Intact rat[c] Feces	Biliary fistulated rat Bile	Biliary fistulated rat Urine	Biliary fistulated rat Feces
Carbaryl ring-[^{14}C][a]	12	0.9	57.1	0.1	0.4	0.3
	24	1.2	82.7	0.2	0.8	32.2
	48	1.3	99.5	0.3	1.3	69.0
Carbofuran ring-[^{14}C][a]	12	4.5	36.5	0.4	0.0	0.0
	24	6.5	67.6	0.7	1.6	10.8
	48	8.0	78.8	1.4	4.4	44.2
Croneton ring-[^{14}C][b]	12	8.2	27.2	0.5	3.4	0.0
	24	14.8	51.2	1.7	9.2	12.6
	48	16.4	90.8	2.5	15.2	35.0

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[a] Unextractable metabolites from bean plants administered as a dietary supplement. Values for intact rats are the mean of four animals.

[b] Unextractable metabolites from sorghum administered as a dietary supplement.

[c] Respiratory gases were monitored for $^{14}CO_2$, but none was detected.

excreted in the bile of biliary fistulated rats. A similar pattern of elimination was seen when the UM of Croneton from sorghum was fed to rats. After 2 days 90.8% of the dose was found in the feces and 16.4% in the urine, while the bile of a bile duct-cannulated rat contained only 2.5% of the dose. Thus, the UM were bioavailable only to a slight extent.

Monitoring biliary radiocarbon was a vital aspect of these studies, since the relative quantities of urinary and fecal elimination of foreign compounds is not a good indication of what is or is not absorbed from the gut (Kleassen, 1975). For example, Houston *et al.* (1975) demonstrated that 6 hours after a single I.V. dose of carbaryl carbonyl-[^{14}C] to rats, up to 40% of the administered radiocarbon was excreted into the bile when the common bile duct was cannulated. As shown with intact rats, though, only 3% of an I.V. dose is eventaully eliminated in the feces. On the other hand, from an unpublished study on α endosulfan, we found that only 14% of an oral dose was eliminated in the urine in 48 hours, while 35% was voided via the feces. These data would lead one to conclude that α endosulfan is slowly absorbed from the gastrointestinal tract. However, up to 58% of an oral dose was found in the bile of bile duct cannulated rats in the same time period. Other examples of this same phenomena are the polycyclic hydrocarbons 1:2-benzanthracene and 3,4-benzopyrene, which undergo extensive biliary excretion and are eventually eliminated in the feces (Falk 1963, Boyland and Sims 1964, Levine 1970).

It should be noted in Table 53 relative to the elimination of UM that the total excretion of Croneton UM from intact rats in 48 hours was 107% of the dose, for carbaryl UM, 100%, and for carbofuran UM, 86%. In contrast, only 50%, 71%, and 50% of the respective UM dose was eliminated by biliary fistulated rats in the same time period. When the study on a bile duct-cannulated rat treated with carbaryl UM was extended to 3 days, the total elimination of radiocarbon increased to 99.3% of the dose, or an amount equivalent to that observed in intact rats in 2 days. This slower elimination seems to be a characteristic attributable to the surgical manipulations of the animal. Such a conclusion is supported below by a study on the fate of carbaryl WSM in rats.

Six rats were treated with carbaryl WSM; one with the bile duct cannulated, one sham-operated, and four intact. The results are shown in Table 54. The four intact rats gave very similar results as well as good radiocarbon accountability. After 24 hours, about 85% of the dose was found in the urine and 7% in the feces. However, the sham-operated and cannulated rats demonstrated a much slower elimination capacity in the first 24 hours with total excretion amounting to 51 and 35% of the dose, respectively. This investigation was carried out to 66 hours post-treatment, and by the end of this period the total elimination of carbaryl WSM for the three types of rats had drawn quite close together. For the bile duct cannulated, sham, and intact rats the recoveries were 89.2, 94.6, and 99.6% of the dose. Thus, surgical manipulation of rats obviously decreased the rate of elimination of carbaryl WSM, although the vast majority of radiocarbon is eventually excreted within a 3 day period as is normally seen with intact rats.

To further investigate these effects on excretion kinetics due to surgery, a slightly different approach was taken when six rats were treated with aldicarb WSM. As with the data discussed above, there was one biliary fistulated rat, one sham-operated and four intact, but dosing of the "operated" rats was delayed until 4 hours post-surgery. As can be seen in Table 54, only in the first 12 hours post-treatment is there any noticeable differences in the total amount of radiocarbon excreted. At this time the intact rats had eliminated 78.8% of the dose, while that from the sham-operated amounted to 23.6%, and for the bile duct cannulated 64.1%. By 24 hours the respective quantities were 86.8, 69.0, and 79.0%. These data on the fate of aldicarb WSM in rats and that on carbaryl WSM tend to support the conclusion that the slower elimination observed in surgical animals is caused by a transient re-

Table 54. ELIMINATION OF RADIOCARBON FROM RATS TREATED WITH CARBARYL, ALDICARB AND CARBOFURAN WATER-SOLUBLE METABOLITES FROM BEAN PLANTS

		Cumulative % of dose							
		Intact rat[b]			Biliary fistulated rat			Sham operated rat	
Water-soluble metabolites[a]	Hours after treatment	$^{14}CO_2$	Urine	Feces	Bile	Urine	Feces	Urine	Feces
Carbaryl ring-[^{14}C][c]	6	0.0	45.8	0.0	0.6	0.0	0.0	0.0	0.0
	12	0.0	74.4	2.1	1.5	0.0	0.0	20.0	0.1
	24	0.0	85.4	7.4	7.1	30.0	1.1	51.1	0.1
	36	0.0	87.7	9.7	12.9	64.4	5.1	74.6	13.2
Aldicarb thiomethyl-[^{14}C][d]	6	0.4	50.0	0.2	11.6	22.5	1.1	0.0	0.0
	12	0.6	78.2	0.6	17.3	45.5	1.3	23.6	0.0
	24	0.6	85.4	0.8	20.0	57.3	1.7	68.4	0.6
	36	0.6	87.5	1.0	20.7	58.9	1.7	78.0	0.6
Carbofuran ring-[^{14}C][c]	6	0.0	28.4	0.0	0.9	0.0	0.3		
	12	0.0	55.5	3.6	2.6	2.9	0.5		
	24	0.0	63.0	6.0	11.0	36.6	1.3		
	36	0.0	64.8	8.3	17.2	55.8	2.1		

[a] Administered via oral intubation.

[b] Mean data from four rats.

[c] Surgically altered animals treated immediately after operation.

[d] Surgically altered animals treated 4 hours after operation.

covery period immediately following the operation, and not factors at play throughout the entire test period. The effect is possibly due to decreased absorption caused by a temporary loss of gastrointestinal motility following surgery. Gastrointestinal motility markedly affects absorption of substances from the gut (Parsons 1975).

It is evident from the rat excretion data in Table 54 and 55 that WSM of the four carbamate insecticides were readily available for absorption, since the bulk of oral doses was recovered in the urine. In 36 hours the urinary radiocarbon of intact rats accounted for 87.7, 87.5, 81.2, and 64.8% of the WSM dose for carbaryl ring-[^{14}C], aldicarb thiomethyl-[^{14}C], Croneton ring-[^{14}C], and carbofuran ring [^{14}C]. Feces contained 9.7, 1.0, 3.7, and 8.3% of the dose, respectively. In the same time period, bile of biliary fistulated rats treated with WSM of aldicarb, carbofuran, and carbaryl comprised of 20.7, 17.2, and 12.9% of the dose. The quantity of aldicarb WSM radiocarbon found in the bile after 36 hours is about ten times greater than the amount eliminated in the feces of an intact rat in 72 hours (2.2% of the dose). This suggests an enterohepatic circulation of these metabolites. Even at that, however, aldicarb WSM as well as those of the other insecticides studied are rapidly eliminated from rats.

Since the WSM are bioavailable, the chemical nature of these metabolites and their fate in rats prior to elimination becomes an important toxicological question. Moreover, when WSM of Croneton carbonyl-[^{14}C] were administered orally to rats (Table 55) 30.0% of the dose was found as $^{14}CO_2$, 39.2% in the urine and 6.1% in the feces. These data from a carbonyl label demonstrated that WSM with an intact carbamate moiety were readily capable of being absorbed. Such an observation lends even more weight to the need for defining sequential biochemical pathways of these insecticides from plants through animals. Thus, an analysis of the WSM and UM from treated bean plants and the urinary radiocarbon from rats administered these metabolites was undertaken.

Table 55. ELIMINATION OF RADIOCARBON FROM RATS TREATED WITH CRONETON WATER-SOLUBLE METABOLITES FROM BEAN PLANTS

Water-soluble metabolites[a]	Hours after treatment	Cumulative % of dose[b]		
		$^{14}CO_2$	Urine	Feces
Croneton ring-[^{14}C]	6	0.0	66.6	0.0
	12	0.0	76.6	0.2
	20	0.0	78.8	2.8
	26	0.0	79.8	3.4
	30	0.0	80.7	3.6
	36	0.0	81.2	3.7
Croneton carbonyl-[^{14}C]	6	23.1	31.2	0.1
	12	30.0	34.5	0.2
	18	30.0	37.5	3.8
	24	30.0	38.4	5.6
	30	30.0	39.0	5.7
	36	30.0	39.2	6.1

[a] Administered via oral intubation.

[b] Mean data from three rats.

The WSM of all four insecticides were treated with glucosidase, since the major conjugating mechanism in plants is glucoside conjugation. Based on the enzyme treatment, the glucosides of carbaryl, carbofuran, aldicarb, Croneton ring-[^{14}C], and Croneton carbonyl-[^{14}C] amounted to 29, 39, 14, 51, and 15% of the WSM, respectively.

Attempts to release the UM from the bean plant solids using acid hydrolysis was, for the most part, unsuccessful. The radiocarbon released as chloroform soluble amounted to only 1.2, 0.7, 0.4, 0.3, and 0.2% of the dose for carbaryl, carbofuran, aldicarb, Croneton ring-[^{14}C], and Croneton carbonyl-[^{14}C], respectively.

The exocons tentatively identified by tlc analysis from glucosidase treatment of the WSM and acid hydrolysis of the plant solids are presented in Table 56. The majority of the Croneton ring-[^{14}C] equivalents released by glucosidase were hydrolytic products, primarily Croneton phenol sulfoxide (52.4% of the exocons) and phenol sulfone (39.8%). The glucosidase released exocons shown with Croneton carbonal-[^{14}C] in Table 56 are, of course, only products with the carbamate intact. Hydrolysis of the carbamic acid ester with carbonyl [^{14}C] carbamates results in loss of the radioisotope as $^{14}CC_2$. The glucosides of Croneton carbonyl-[^{14}C] amounted to only 0.2% of the dose, while the glucosides of Croneton ring-[^{14}C] were 24.6%. Overall, as evidenced by these results on the two [^{14}C]-labels, glucosidation of Croneton was predominantly with the phenolic metabolites.

With aldicarb 80% of the glucosidase released radiocarbon remained at the origin of tlc plates (Table 56). The two identified metabolites were aldicarb sulfoxide (7.5% of the radioactivity present), and oxime sulfoxide (5%). When the carbaryl glucosidase released exocons were analyzed by tlc, 5.8, 7.0, 12.8, 11.6, and 3.5% of the radiocarbon present was identified as N-hydroxymethyl carbaryl, 4-hydroxy carbaryl, 5-hydroxy carbaryl, carbaryl, and 1-naphthol. When carbofuran WSM were treated with glucosidase, the exocons released and

Table 56. THIN-LAYER CHROMATOGRAPHY OF RADIOCARBON RELEASED BY ACID AND ENZYME HYDROLYSIS OF THE BOUND AND WATER-SOLUBLE RESIDUE FROM BEAN PLANTS TREATED WITH CARBAMATE INSECTICIDES

	% of radiocarbon released	
	Water solubles	Unextractables
Insecticide/Metabolite	Glucosidase released	Acid hydrolyzed
Carbaryl ring-[^{14}C]		
Origin	36.0	54.2
3,4-dihydroxy	0.0	0.0
5,6-dihydro-dihydroxynaphthol	0.0	13.3
N-hydroxymethyl	5.8	0.0
4-hydroxy	7.0	0.0
5-hydroxy	12.8	0.0
parent	11.6	0.0
1-naphthol	3.5	12.5
unknown	23.3	20.0
Carbofuran ring-[^{14}C]		
Origin	1.0	7.2
3-hydroxy	83.0	47.9
3-hydroxyphenol	6.8	30.4
3-keto	0.0	0.0
3-ketophenol	3.9	11.6
phenol	1.9	2.9
unknown	3.4	0.0
Croneton ring-[^{14}C]		
Origin	0.5	3.3
sulfoxide	0.8	20.0
phenol sulfoxide	52.4	46.7
sulfone	0.8	6.7
phenol sulfone	39.8	20.0
parent	1.2	3.3
phenol	0.0	0.0
unknown	4.5	0.0
Croneton carbonyl-[^{14}C]		
Origin	2.4	5.0
sulfoxide	17.1	75.0
sulfone	4.9	15.0
parent	0.0	0.0
unknown	75.6	5.0
Aldicarb thiomethyl-[^{14}C]		
Origin	80.0	11.6
sulfoxide	7.5	0.0
oxime sulfoxide	5.0	34.9
nitrile sulfoxide	0.0	39.5
unknown	7.5	14.0

identified as hydrolytic products were minor in amount as compared to those exocons identified as oxidation products of the insecticide. Eighty-three percent of the radiocarbon cleaved was 3-hydroxy carbofuran. Other exocons found were 3-hydroxy carbofuran phenol (6.8%), carbofuran phenol (1.9%), and 3-keto carbofuran phenol (3.9%).

It is difficult to draw many inferences from the results on tlc of the radiocarbon released by acid hydrolysis of the plant solids (Table 56). Very little of the radioactivity released was organosoluble and, thus, conducive to chromatographic analysis. In general, the principal exocons that were identified from the plant solids were the same as those found with the glucosidase released exocons of the WSM. The major exceptions to this generalization were the aldicarb nitrile sulfoxide amounting to 39.5% of the exocons released from the solids, and the 5,6-dihydro-dihydroxynaphthol, a metabolite from the solids containing carbaryl UM, amounting to 13.3%. Neither of these exocons were identified from among the aglycones of the respective WSM.

Very little of the radiocarbon recovered in the urine of WSM treated rats was organosoluble. Aldicarb was the only insecticide that resulted in any significant partitioning of urinary radiocarbon into chloroform, and this accounted for only 12.7% of the administered WSM. The other four groups of WSM treated animals had considerably less organosoluble urinary radiocarbon. The values were 2.3, 2.5, 1.0, and 0.4% of the dose for carbaryl, carbofuran, Croneton ring-[^{14}C], and Croneton carbonyl-[^{14}C], respectively. Thus, there were very few free metabolites in the urine from any of the animals administered WSM, but such results are not surprising when one considers the water soluble nature of the inoculum.

Enzyme hydrolysis (glucosidase, glucuronidase, and sulfatase) of this urine released radiocarbon that was organoextractable in amounts equal to 45.1, 23.3, 11.7, 46.8, and 11.7% of the dose for carbaryl, carbofuran, aldicarb, Croneton ring-[^{14}C], and Croneton carbonyl-[^{14}C], respectively. When urine from WSM treated rats was subjected to an acid hydrolysis, more exocons were released than that resulting from enzyme cleavage, except for urine containing Croneton equivalents. Specifically, 47.8, 42.0, 19.9, 31.8, and 2.0% of the WSM dose was released by acid hydrolysis of urine containing equivalents of carbaryl, carbofuran, aldicarb, Croneton ring-[^{14}C], and Croneton carbonyl-[^{14}C], respectively. Acid hydrolysis of the Croneton carbonyl-[^{14}C] urine resulted in a loss of radiocarbon equivalent to 23.2% of the dose. Presumably, the loss of radioactivity from this urine is the result of released volatile products which escaped into the atmosphere.

Table 57 shows the results of a tlc analysis undertaken on exocons released in the acid and enzyme hydrolysis of rat urine discussed above. No data on Croneton carbonyl-[^{14}C] is presented in this table, because attempts to identify the low levels of radiocarbon released by acid and enzyme hydrolysis of rats treated with the WSM were unsuccessful. The entire dose to rats amounted to only 110,000 dpm.

From the urine containing carbofuran equivalents, three major exocons were found that accounted for 90% or more of the radiocarbon released by the three enzyme treatments and acid hydrolysis. These were 3-hydroxy carbofuran, 3-hydroxy carbofuran phenol, and 3-keto carbofuran phenol.

1-Naphthol was the major exocon identified from the urine of rats treated with carbaryl WSM, amounting to over 50% of the combined radiocarbon present as glucosides, glucuronides, sulfates, and acid hydrolyzed metabolites. From the glucosidase released carbaryl exocons out of rat urine, 25% was identified as 5,6-dihydro-5,6-dihydroxy carbaryl, and 29, 17, and 13% of the sulfates were identified as 5-hydroxy carbaryl, 3,4-dihydroxy carbaryl, and 5,6-dihydro-5,6-dihydroxy carbaryl, respectively. These were minor metabolites, though,

Table 57. THIN-LAYER CHROMATOGRAPHY OF RADIOCARBON RELEASED BY ACID AND ENZYME HYDROLYSIS OF URINE FROM RATS TREATED WITH THE WATER SOLUBLE RESIDUE OF CARBAMATE INSECTICIDES FROM BEAN PLANTS

	% of radiocarbon released by			
Insecticide/Metabolite	Glucosidase	Glucuronidase	Sulfatase	Acid
Carbaryl ring-[^{14}C]				
Origin	25.0	2.5	10.2	35.4
3,4-dihydroxy	0.0	0.0	17.4	0.0
5,6-dihydro-5,6-dihydroxy	0.0	0.0	13.0	0.0
5,6-dihydro-dihydroxynaphthol	25.0	0.0	0.0	0.0
4-hydroxy	0.0	0.0	0.0	5.6
5-hydroxy	0.0	0.0	29.0	3.1
1-naphthol	50.0	90.1	30.4	50.4
unknown	0.0	7.4	0.0	5.5
Carbofuran ring-[^{14}C]				
Origin	2.3	0.8	13.8	8.3
3-hydroxy	20.6	4.1	29.5	6.7
3-hydroxy phenol	54.4	8.3	29.5	8.3
3-keto phenol	13.9	78.5	22.7	70.7
phenol	5.9	5.0	0.0	0.0
unknown	2.9	3.3	4.5	6.0
Croneton ring-[^{14}C]				
Origin	0.9	2.2	3.8	1.9
sulfoxide	0.0	0.0	0.0	24.5
phenol sulfoxide	0.0	46.9	0.0	67.6
phenol sulfone	41.7	0.0	0.0	6.0
phenol	0.0	0.0	46.2	0.0
unknown	57.4	50.9	50.0	0.0
Aldicarb thiomethyl-[^{14}C]				
Origin	57.7	12.7	10.8	15.6
sulfoxide	42.3	22.2	25.0	0.0
oxime sulfoxide	0.0	17.5	46.4	47.7
sulfone	0.0	0.0	7.1	0.0
nitrile sulfoxide	0.0	7.9	3.6	36.7
oxime sulfone	0.0	0.0	7.1	0.0
unknown	0.0	39.7	0.0	0.0

because all the glucosides and sulfates combined from the carbaryl WSM treated rats amounted to only 9% of the dose.

Aldicarb sulfoxide and oxime sulfoxide accounted for 60% of the radiocarbon released by the three enzyme treatments and acid hydrolysis of urine from the aldicarb WSM treated rats. About 37% of the acid hydrolyzed exocons was the aldicarb nitrile sulfoxide, whereas only a small quantity of this metabolite, amounting to less than 1% of the dose, was isolated following enzyme treatments of the urine. It is possible that the oxime sulfoxide was converted to the nitrile sulfoxide under acid conditions.

Croneton sulfoxide, phenol sulfoxide, and phenol sulfone were the major exocons identified from the urine of Croneton ring-[^{14}C] WSM treated rats. Although 46% of the sulfates was the Croneton phenol, this was equivalent to only 1.3% of the dose, and could not be considered a major identified product. The combined glucosides and glucuronites accounted for 44.2% of the dose, thus the identified products resulting from these two enzyme treatments were a better indication of the major exocons present in rat urine. No Croneton sulfoxide was found following any of the enzyme treatments of urine, but 24.5% of the acid released exocons were so identified, amounting to 7.8% of the dose.

The tentatively identified exocons from rat urine with the carbamic acid ester intact were 3-hydroxy carbofuran (9.2% of the enzyme and acid released radiocarbon combined) 3,4-dihydroxy carbaryl (1.3%), 5,6-dihydro-5,6-dihydroxy carbaryl (1%), 4-hydroxy carbaryl (2.9%), 5-hydroxy carbaryl (3.8%), aldicarb sulfoxide (10.1%), aldicarb sulfone (0.6%), and Croneton sulfoxide (9.9%). With the exception of 3,4-dihydroxy carbaryl, 5,6-dihycro-5,6-dihydroxy carbaryl, and aldicarb sulfone, all of these carbamate exocons were found in the WSM from bean plants (Table 57) in equal or greater amounts than in the urine of rats treated with the WSM. In only three instances were the intact carbamate exocons a relatively significant proportion of the enzyme and acid released urinary radiocarbon. These were 3-hydroxy carbofuran, aldicarb sulfoxide, and Croneton sulfoxide. For the most part, then, rats degraded the potentially toxic plant conjugates. The majority of radiocarbon released by enzyme and acid hydrolysis of urine was phenolic derivatives, unknowns, and polar origin material with all four insecticides studies.

From the data discussed above it is apparent that upon absorption of WSM by rats, the metabolic degradation of the insecticides begun in bean plants was continued. This was most clarly demonstrated in the carbofuran study. In plants treated with that insecticide, a predominance of oxidation was seen at the three position of the benzofuranyl ring to form 3-hydroxy carbofuran (Table 56). This metabolite accounted for 83% of the aglycones, while phenolic products of carbofuran amounted to only about 13%. After administration of the carbofuran WSM to rats, only 9.2% of the urinary exocons was 3-hydroxy carbofuran, whereas 14% was the 3-hydroxy carbofuran phenol and 63% the 3-keto carbofuran phenol. Furthermore, almost 21% of the carbofuran WSM were glucosides, but when the WSM were administered to rats only 6.8% of the dose was eliminated in the urine as glucosides. Glucuronides and sulfates amounted to 16.5% of the dose. Presumably from this data, there was cleavage of 3-hydroxy carbofuran glucoside within the animal, followed in some order by oxidation of the 3-hydroxy to a keto, hydrolysis of the carbamate, and conjugation with either glucuronic or sulfuric acid prior to elimination.

It is difficult to make assessments, such as the one above, based solely on enzyme analyses since enzyme preparations are not altogether specific. However, a study utilizing a naphthyl-[^{14}C] glucoside preparation and a naphthyl glucoside-[^{14}C] label showed that only 20% of the conjugate was excreted intact as administered, while 24% was eliminated as a glucuronide (Dorough *et al.* 1974). The use of two [^{14}C] labels enabled these workers to ascertain that the glucuronide was not formed by oxidation of 1-naphthyl-glucoside, but resulted from the cleavage of the glucoside linkage to form 1-naphthol, which was then conjugated with endogenous glucuronic acid. It was demonstrated that the administration of free 1-naphthol, a metabolite of carbaryl, resulted in a more rapid excretion than its glucoside. This suggests that the cleavage occurs in the gut since the water soluble nature of a glucoside would be a deterrent to absorption.

When the carbaryl WSM were administered to rats only 3.5% of the aglycones in the dose was 1-naphthol (Table 57). However, based on enzyme and acid hydrolysis of urinary radiocarbon, almost 60% of the dose was eliminated as conjugates of 1-naphthol, and less than 2% of these were glucosides. A continuing degradative process of WSM in the rat is readily apparent. In contrast, little difference was seen between the aglycones of Croneton ring-[^{14}C] WSM and the exocons of urine from rats treated with these WSM. In both cases, the Croneton phenol sulfoxide and phenol sulfone were the major identified metabolites. Unlike the other carbamates, Croneton was quickly hydrolyzed by bean plants, and this would account for the similarity of products found in plants and rats.

Not many inferences can be drawn on data generated with aldicarb, because so little of the WSM dose (4%) in plants was cleaved by glucosidase, and 80% of this remained on the origin of tlc plates. The major identified aglycones of the WSM were aldicarb sulfoxide and oxime sulfoxide. Similarly, these were found in high quantities among the exocons of urine from rats administered aldicarb WSM at 10.1 and 37.6% of the radiocarbon present, respectively.

An analysis of the urine from rats administered carbaryl and carbofuran UM resulted in just 0.7 and 1.1% of the dose partitioning into chloroform, respectively. Enzyme treatments freed 19% of the radiocarbon in the urine of the rat fed carbofuran UM, and 17% of that in the urine of carbaryl UM treated rats. Acid hydrolysis was more effective in releasing exocons from urine, but this still amounted to only 4.9% of the carbofuran UM dose, and of 0.5% of the carbaryl UM dose. Thin layer chromatography on exocons released from the urine of carbaryl and carbofuran UM treated rats was inconclusive, due to the low radioactivity and large amount of interfering material extracted with the exocons. When carbofuran and carbaryl plant solids were treated with acid, 10% and 2% of the radiocarbon present was released into chloroform, respectively. These are roughly the same percentages of bound residues administered to rats that were recovered in the urine, and might be the same metabolites available for absorption. Thin layer chromatography of the acid released exocons from the plant solids (Table 56) showed that 3-hydroxy carbofuran was the only liberated product with intact carbamate. The remaining were phenolic derivatives and unidentified materials with both the insecticides. However, it has been reported that six days after a stem injection to bean plants of carbaryl carbonyl-[^{14}C], 35% of the radiocarbon was bound to plant solids (Abdel-Wahab *et al*. 1966). The data indicated that the majority of the carbaryl unextractables were intact carbamate. Aside from the tlc mentioned above, support of this conclusion is evident from the results of the present study using the ring label, where a similar quantity of UM were detected. Likewise, Kuhr and Casida (1967) obtained evidence that the UM were carbamate-type metabolites. No sound conclusions can be drawn on the nature of the small quantities of carbaryl and carbofuran UM that were available for absorption.

Two main processes govern the absorption of drugs and foreign compounds from the gastrointestinal tract (Aguiar 1975). One is the build-up of an effective concentration of the chemical in solution at the absorption site, and the other is the rate of permeation through the gastrointestinal barrier. When a compound is of an insoluble nature, such as the UM investigated, the accumulation of an effective concentration at the site governs the rate of absorption. It was evident from a close examination of the feces from rats treated with UM that little physical change had taken place in the plant solids containing the UM during their course through the alimentary tract. This lack of dissolution of the administered material helps explain the unavailability of the UM studied.

On the other hand, when drugs or foreign compounds are administered in solution, as was the case with the WSM, the permeation rate is assumed to be the controlling step in the absorption sequence (Aguiar 1975). Permeation of the WSM was rapid and extensive as demonstrated by the urinary excretion data on rats treated with these metabolites. Since rapid absorption of a chemical is generally associated with a high lipid solubility (Parke 1968), the bioavailability of the very polar carbamate insecticide WSM appears to be a complex phenomena. Gastrointestinal motility, physiological and metabolic properties of the intestinal epithelia, and presence of gastrointestinal microflora certainly could all play a significant role (Kurz 1975). An investigation of these parameters and their contribution to the bioavailability of carbamate insecticide WSM would be of toxicological interest.

FURTHER STUDIES ON THE CHEMICAL NATURE OF RADIOACTIVE WATER SOLUBLE AND BOUND RESIDUES IN PLANTS TREATED WITH ^{14}C-CARBARYL

Materials and Methods

Chemicals - 1-Naphthol-1-^{14}C (15.2 mCi/mM) was obtained from Amersham Searle Company, Des Plaines, IL., while naphthalene-^{14}C (5.1 mCi/mM) was purchased from California Bionuclear Corporation, Sun Valley, CA. Nicotamide adenine dinucleotide phosphate ($NADPH_2$), uridine-5-diphosphoglucose (UDPG), tris(hydroxymethyl)aminomethane (Tris), and β-D-glucoside glucohydrolase (EC No. 3.2.1.21) were supplied by Sigma Chemical Company, St. Louis, MO. Triethyl amine was obtained from Matheson, Coleman and Bell, Chicago, IL.

Synthesis - Radiolabeled carbaryl (naphthyl-1-^{14}C-carbaryl) 15.2 mCi/mM) was synthesized essentially by the procedure of Dorough and Casida (1964) except that triethylamine was added as a catalyst to the reactants 1-naphthol-1-^{14}C and methyl isocyanate, to increase the yield. 1-Naphthyl-1-^{14}C-glucoside was prepared with crude fly homogenate using the procedure of Kumar and Dorough (1974).

1-Methoxynaphthalene-1-^{14}C was prepared from 1-naphthol-1-^{14}C by the diazomethane procedure of Pyrek and Achmetowicz (1970). All of the above radiolabeled compounds were purified by thin-layer chromatography on silica gel plates (Brinkman 254, 0.24 mm). The preparation of 1-naphthyl glucoside has previously been described (Dorough *et al.* 1974). The intermediate 1-naphthyl tetra-O-acetyl-β-D-glucopyranoside was characterized by its nmr (acetone d_6), δ2.00 (S, 12H, acetyl CH), δ2.78 (d, 1H aliphatic CH), δ4.19 (m, 2H, CH), δ5.20-5.58 (m, 4H aliphatic-CH) and δ7.0-8.0 (m, 7H aromatic CH). The melting point of the naphthyl glucoside was 171-175°C.

Generation of Carbaryl Metabolites by Plants - Dwarf Horticulture (Taylor) bush beans were grown in the greenhouse in sterilized soil to reduce microbial growth during the summer months in the greenhouse, and transferred to environmental growth chambers 9 days after germination. The plants were watered daily with Hoaglands nutrient solution and kept at 27°C during the day and 23°C at night with a 12-hr photo period. Treatment occurred when the plants were 12 days old. Twenty-two µg of naphthyl-1-^{14}C-carbaryl were injected in 75 µl as a 3:1 water-acetone solution into the stem above the soil level. The stem was punctured 2 cm above the point of injection in order to prevent back pressure during injection.

To extract radioactive compounds individual plants were homogenized in a Virtis 45 homogenizer in 75 ml:25 ml acetone-water at high speed for five min. The homogenate was filtered under vacuum using a Buchner funnel and Whatman No. 4 filter paper. The solids were rehomogenized twice more for 5 min in 40 ml:40 ml:40 ml acetone-methanol-ethyl acetate and filtered. This solvent mixture eliminated soxhlet extraction of the solids, which extracted an additional 5% radioactivity as tried in earlier experiments. The combined filtrates were evaporated until only the water remained, transferred to separatory funnels and washed 3 times with hexane to remove lipids and chlorophyll. The hexane wash contained no radioactivity. The water layer was then extracted three times with 100 ml chloroform to separate the water-soluble metabolites from the parent compound. Both water- and organo-fractions were radioassayed. The total solids containing the "unextractable" metabolites of each plant were combusted for radioassay in 2-3 portions at 95% efficiency on a Packard 306 Tricarb Oxidizer.

Aglycones from plant-generated ^{14}C-carbaryl water soluble metabolites were prepared by adjusting the water soluble carbaryl metabolites to 1.0 N HCl and heating for 1 hr in a boiling water bath. After cooling, 2 ml of saturated NaCl in water were added to the 4 ml reaction and extracted 4 times with 10 ml of chloroform. The chloroform was evaporated and the aglycones dissolved in 3:1 water:acetone prior to treatment of bean plants or bean plant homogenates.

In order to generate aglycones by enzyme hydrolysis, carbaryl water solubles were freeze-dried and resuspended in 10 ml of 1 M acetate buffer, pH 5. This solution, containing approximately 6,200,000 dpm of ^{14}C-carbaryl water-solubles was transferred to a 25 ml Erlenmeyer flask and placed into a water bath shaker at 37°C. Two mg of β-D-glucoside glucohydrolase in 2 ml of buffer were added and incubated for 24 hr. A control containing about 200,000 dpm of water solubles was set up in the same manner without the enzyme. After terminating the reaction with chloroform, the aqueous phase was extracted 4 times with 50 ml chloroform. Thirty ml of saturated sodium chloride solution were added prior to extraction.

In vitro Metabolism - Ten grams of 12-day-old bean plants were cut into small pieces, sealed into a plastic bag and quick frozen in dry ice-acetone. The plant material was then ground with a pre-frozen mortar and pestle to a very fine powder and suspended up to 100 ml with 0.5 M phosphate buffer, pH 7.2, containing 0.15 M KCl. In one experiment with ^{14}C-carbaryl NADPH (1 mg/ml reaction mixture) and NaCN (25 μg/ml) were added in an attempt to increase the metabolism of plant enzymes and to inhibit phenol oxidases, respectively.

The suspended plant homogenate was stirred on a magnetic stirrer until equilibrated to 25°C. The radiolabeled compound was then added immediately while stirring continuously. Ten ml aliquots were taken after the following time intervals: 1 min, 2.5 min, 15 min, 30 min, 1 hr, and 2 hr. The aliquots were pipetted into 500 ml separatory funnels containing 30 ml of distilled water and 100 ml of chloroform. After shaking and separating the two phases, the water layer was extracted twice more with 100 ml of chloroform. The combined organo extracts were evaporated to a low volume, transferred to graduated centrifuge tubes and radioassayed by liquid scintillation. The plant emulsion which occurred during extraction was separated by centrifugation. The plant solids were added back to the water layer remaining in the separatory funnel and 100 ml of acetone were added, shaken, and allowed to precipitate for 15 min. The solutions were then filtered under vacuum, the solids washed with 1:1:1 H_2O-acetone-ethyl acetate. After evaporation of the solvents, the water layer was radioassayed and the solids combusted. The following radiolabeled compounds were subjected to this *in vitro* system: ^{14}C-carbaryl (15.2 mCi/mM); 1-naphthol-1-^{14}C (15.2 mCi/mM), 1-naphthol-1-^{14}C-glucoside (15.2 mCi/mM); naphthalene-^{14}C (5.1 mCi/mM) and 1-methoxynaphthalene-^{14}C (15.2 mCi/mM).

Results and Discussion

The distribution of carbaryl equivalents in 12-day-old bean plants 0 to 25 days after stem injection with 1 μg of naphthyl-1-^{14}C-carbaryl is shown in Table 58. ^{14}C-Carbaryl equivalents were rapidly distributed between the water soluble and solid phases one day after treatment with a greater proportion of the dose being incorporated into the water soluble phase. The distribution of radiolabel in the two phases remained essentially the same until day 15 post-treatment at which time there was a noticeable decline in water soluble metabolites at the expense of an increase in "bound" metabolites. This trend continued for the remainder of the experiment. Earlier studies by Dorough and Wiggins (1968) had shown a similar redistribution of ^{14}C-carbaryl metabolites in bean plants 10-20 days after treatment with 25 μg ^{14}C-carbaryl. This suggested that water soluble metabolites, possibly the glycoside conjugates of phase I carbaryl metabolism, were the immediate precursors to unextractable metabolites (Kuhr and Dorough, 1976). However, the distribution data from plants treated with 1 μg of carbaryl indicate that another mechanism for formation of unextractable metabolites may exist. This was indicated by the rapid incorporation of radiolabel into the water-soluble and "unextractable" plant fractions one to two days after treatment followed by a period of 10-12 days with essentially no change in residue levels in the two fractions. As indicated in Table 58, injecting plants with 25 μg ^{14}C-carbaryl effectively masks the distribution pattern seen at the lower dose.

Table 58. EXTRACTION AND PARTITIONING CHARACTERISTICS OF CARBARYL AND METABOLITES AFTER INJECTION INTO BEAN PLANTS

Treatment and fraction	% Distribution/days after treatment									
	0	1	3	5	7	9	11	16	20	25
^{14}C-Ring carbaryl										
1 µg/plants										
Organosolubles	98	8	7	7	12	0	0	0	0	0
Water-solubles	0	62	57	56	58	61	62	50	30	25
Bound	2	30	36	37	30	39	38	50	70	75
25 µg/plant										
Organosolubles	95	56	40	21	10	2	-	-	-	-
Water-solubles	1	13	33	45	49	52	-	-	-	-
Bound	4	21	27	34	41	46	-	-	-	-
^{14}C-Naphthol										
25 µg/plant										
Organosolubles	84	8	9	7	0	-	-	-	-	-
Water-solubles	10	13	14	11	8	-	-	-	-	-
Bound	6	79	77	82	92	-	-	-	-	-
^{14}C-Naphthylglucoside										
1 µg/plant										
Organosolubles	0	10	15	14	14	18	25	35	-	-
Water-solubles	95	48	54	60	61	60	55	50	-	-
Bound	5	42	31	26	25	22	20	15	-	-

The rapid incorporation of ^{14}C-carbaryl equivalents into the unextractable fraction the first one to two days after treatment suggested to us that the phase I, hydroxylated carbaryl derivatives were the proximate intermediates in the formation of "bound" residues and not the conjugated derivatives of phase I products. More specifically, the formation of conjugated and "bound" metabolites proceeds by two different pathways each competing for a common substrate, e.g., hydroxylated carbaryl derivatives.

If hydroxylated derivatives are proximate intermediates in the formation of "bound" residues, then administering these radiolabeled materials to plants should lead to a more rapid and extensive incorporation of radioactivity into the "bound" residues than would be the case if water-soluble metabolites were administered to the plants. Indeed, when 12-day-old bean plants excised at ground level were allowed to take up carbaryl aglycones (acid released from ^{14}C-ring-carbaryl water soluble metabolites obtained from carbaryl treated bean plants), there was rapid and extensive incorporation of radiolabel into the "bound" fraction and water soluble fraction as indicated by the data in Table 59. It was also observed that 1-naphthol-1-^{14}C, a phase I metabolic product of ^{14}C-carbaryl, was rapidly and extensively incorporated into unextractable fraction after stem injection with only about 10% of the applied dose being distributed to the water soluble phase (Table 58). The results with naphthol are consistant with those obtained by Kuhr and Casida (1967).

When excised bean plants were allowed to take up water soluble metabolites derived from carbaryl treated plants, only a small fraction of the applied radioactivity was incorporated into the unextractable fraction even after 25 days post-treatment as shown by the data in Table 59. Stem injecting bean plants with 1 μg of 1-naphthol-1-^{14}C-glucoside, a water-soluble carbaryl metabolite, lead to a distribution pattern different from that obtained with the total crude water soluble metabolites. As indicated in Table 58, there was a rapid decrease in radioactivity associated with the water-soluble fraction and a corresponding increase

Table 59. EXTRACTION CHARACTERISTICS OF RADIOCARBON IN EXCISED BEAN PLANTS FOLLOWING UPTAKE VIA THE STEM OF CARBARYL WATER-SOLUBLE METABOLITES (BEAN PLANT-GENERATED) AND AGLYCONES RESULTING FROM ACID HYDROLYSIS

Treatment and fraction	% Distribution/days after treatment					
	4	5	8	10	20	25
^{14}C-Ring-Carbaryl water soluble metabolites						
Organosolubles	15	14	13	10	10	11
Water-solubles	82	74	71	82	78	76
Bound	3	12	16	8	12	13
^{14}C-Aglycones[a]						
Organosolubles	17	12	5	6	-	-
Water-solubles	55	40	48	61	-	-
Bound	28	48	47	33	-	-

[a] ^{14}C-Metabolites cleaved from carbaryl watersolubles from bean plants by acid treatment.

in the radiolabel associated with the unextractable phase. Also, there was a gradual increase in radioactivity associated with the organo-soluble fraction. The nature of the organo-soluble fraction is unclear. The data would suggest something other than 1-naphthol, since, as we have seen previously (Table 58), injecting 1-naphthol-1-^{14}C into bean plants leads to almost complete incorporation of the radiolabel into the unextractable phase and to a much lesser extent into the water-soluble phase. The preceeding results obtained with carbaryl water-soluble metabolites, their acid-released aglycones, and 1-naphthol indicate that phase I or hydroxylated carbaryl metabolites serve as proximate intermediates to unextractable metabolites. However, it could be argued that the lack of incorporation of water soluble-metabolites into bound residues, the strongest evidence for their not being considered as proximate intermediates to this fraction, may be due to the inaccessibility of these applied metabolites to the cellular sap. That is, because of their polarity, water soluble metabolites may be unable to penetrate the lipoidal cellular membrane and are thus unavailable for enzymatic or nonenzymatic reactions within the cell. Therefore, an in vitro system, using a bean plant homogenate suspended in phosphate buffer (pH 7.2) and 0.15 M KCl, was used in order to eliminate cellular barriers. Table 60 presents the results obtained in such a system using naphthol-1-^{14}C and ^{14}C-carbaryl aglycones (acid released) as the substrate. A time dependent incorporation of label into the polar and unextractable fraction is evident for both substrates. Heating of the plant homogenate for 30 min at 100°C and cooling to room temperature prior to addition of radiolabeled naphthol effectively blocked its incorporation into the polar and unextractable fractions. The heating process presumably inactivated enzymes involved in the reaction of 1-naphthol-^{14}C, although it is possible that reactive sites not necessarily involving enzymes may have been changed due to this drastic treatment. THe rather large percentage (34%) of the ^{14}C-carbaryl aglycone equivalents associated with the polar phase 1.0 min after addition of the radiolabel suggests that some reaction other than enzymatic is involved with this substrate. It is also interesting to

Table 60. *IN VITRO* METABOLISM OF CARBARYL AND RELATED COMPOUNDS BY BEAN PLANT HOMOGENATES

	% Distribution/min of incubation					
Substrate and fraction	1	2.5	15	30	60	120
^{14}C-Ring-carbaryl						
Organosolubles	99	99	99	99	98	99
Water-solubles	1	1	1	1	2	1
Bound	0	0	0	0	0	0
^{14}C-Naphthol						
Organosolubles	94	-	93	14	8	-
Water-solubles	2	-	9	54	62	-
Bound	4	-	8	32	30	-
^{14}C-Naphthalene						
Organosolubles	96	97	98	97	95	-
Water-solubles	4	3	2	3	5	-
Bound	0	0	0	0	0	-
^{14}C-Methoxynaphthalene						
Organosolubles	90	-	79	73	82	90
Water-solubles	10	-	21	36	17	10
Bound	0	-	0	1	1	0
^{14}C-Naphthylglucoside						
Organosolubles	11	13	13	10	14	9
Water-solubles	89	87	87	90	86	91
Bound	0	0	0	0	0	0
^{14}C-Carbaryl water-solubles[a]						
Organosolubles	5	4	5	6	5	5
Water-solubles	95	95	94	94	94	94
Bound	0	1	1	0	1	1
^{14}C-Aglycones[b]						
Organosolubles	62	-	64	59	50	-
Water-solubles	34	-	30	34	39	-
Bound	4	-	6	7	11	-

[a] ^{14}C-Ring-carbaryl water-solubles from bean plants injected with carbaryl.

[b] Aglycones from [a] released by acid treatment.

note that the unextractable metabolites did not appear to be formed at the expense of the apolar fraction (^{14}C-carbaryl aglycones). Based on work with the herbicide cisanilide (cis-2,5-dimethyl-1-pyrrolidinecarboanilide), Frear (1967) suggested that the phenolic intermediates in cisanilide metabolism were further oxidized and served as precursors of an insoluble residue fraction in plants. He also suggested that the phenol oxidase system in plants could mediate the formation of these terminal pesticide residues.

The behavior of a number of substrates in the *in vitro* system are summarized in Table 60. The fact that the radiocarbon from 1-naphthyl-^{14}C-glucoside and ^{14}C-carbaryl water soluble metabolites was not incorporated into the unextractable fraction suggests that these materials are not precursors to the bound residues in the intact plant (Table 58). It appears that compounds which do not have a free hydroxyl group (see also naphthalene and methoxynaphthalene, Table 60) do not form significant amounts of bound residues.

That carbaryl metabolism was not occurring in the bean plant homogenate even in the presence of NADPH (1 mg/ml reaction mixture) and NaCN (25 µg/ml) was indicated by the lack of incorporation of radioactivity into the water and solid phases. Sodium cyanide was included in the reaction medium to reduce phenol oxidase activity which may inactivate oxidative enzymes via phenol oxidation products (Frear *et al.* 1969). However, when the chloroform fractions were subjected to thin-layer chromatography, only carbaryl was present. Without initial hydroxylation, the production of conjugates and bound residues obviously does not proceed.

The data obtained thus far indicate that the hydroxylated carbaryl metabolites may be the proximate intermediates in the formation of bound residues initially formed in carbaryl treated bean plants. That such hydroxylated derivatives of pesticides may be the proximate intermediates has been suggested by the work of others. For example, Briggs *et al.* (1974) reported that the p-hydroxyphenyl derivative of carboxin

(5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide). the major metabolite of this fungicide in plants, was bound to the lignin fraction of growing barley plants. Frear and Swanson (1975) reported quantitative differences in the metabolism of the experimental herbicide cisanilide in cell cultures and excised leaves. Cell cultures when compared to excised leaves showed a reduced capacity to form secondary glycoside conjugates and an increased ability to form methanol-insoluble residues. The major metabolic pathway for this herbicide in carrots and cotton was via hydroxylation at the 4-position of the phenyl ring and the 3-position of the pyrrolidine ring. Furthermore, these authors found that carrot cell suspension cultures treated with a radiolabeled aglycone of cisanilide plant metabolism, 2,5-dimethyl-1-pyrrolidine-4-hydroxy-carboxanilide, rapidly incorporated the radiolabel into an extracellular methanol insoluble residue. Preliminary studies indicated that the radioactivity was bound to a carbohydrate protein thought to be associated with the cell wall surface.

RULE OF GLUTATHIONE CONJUGATION IN THE METABOLISM OF CARBARYL IN RATS

While the importance of glutathione conjugation of pesticides has not been fully elucidated, there is evidence that such conjugation may constitute major routes of detoxification. It is known that glutathione is involved in the transfer of alkyl groups during the metabolism of organophosphorus insecticides in animals and insects (Hutson 1972). Moreover, glutathione conjugation of carbaryl is a proposed metabolic route for in vitro studies using rat liver homogenate, although the glutathione conjugate was not isolated (Bend et al., 1971). It was proposed that conjugation began by oxidation of carbaryl across the 5,6-positions of the naphthalene ring to form the epoxide which reacts with glutathione. Then, the glutathoine conjugate was excreted in the bile or subsequently converted to the mercapturic acid. However, the proposed structures of the glutathione and mercapturic conjugates of carbaryl were based solely on indirect evidence.

The purpose of this investigation was to determine the significance of glutathione and mercapturic acid conjugate formation in the detoxification of certain aromatic chemicals in rats. Naphthalene was selected as a model compound and the insecticide carbaryl was studied to see if these very similar compounds undergo the same metabolic conversion to glutathione conjugates.

Materials and Methods

Chemicals - Naphthalene-1-^{14}C, 1-naphthol-1-^{14}C, and glutathione-^{35}S were purchased from Amersham Searle Company (Chicago, IL) while bromobenzene-ring-UL-^{14}C was purchased from California Bionuclear Corp. (Sun Valley, CA). These chemicals had specific activities of 5.1 mCi/mmol, 15.2 mCi/mmol, 25.7 mCi/mmol and 8.75 mCi/mmol, respectively. 1-Naphthyl-1-^{14}C Npmethylcarbamate (1-naphthyl-1-^{14}C carbaryl) was prepared from methylisocyanate and 1-napthol-^{14}C. Deacetoxydibutyltin was used as a catalyst (Nye et al., 1976). The 1-naphthyl-1-^{14}C carbaryl, purified by tlc, had a specific radioactivity of 15.2 mCi/mmol. Cochromatography in two tlc solvent systems showed that the radioactive chemical was pure. The carbonyl-^{14}C label was synthesized by the reaction of 1.5 to 1 ratio of 1-naphthol and methyl isocyanate-CO-^{14}C in dry benzene. Two drops of diacetoxydibutyltine was also used as a catalyst.

For use as metabolite standard for conjugates, S-(1-naphthyl)-glutathione, S-(1-naphthyl)cysteine, and N-acetyl-S-(1-naphthyl)-cysteine (also called 1-naphthyl mercapturic acid) were synthesized by a modification of the methods of Park and Williams (1951) and Booth et al. (1960) in which diazotied 1-naphthylamine and cuprous mercaptide were utilized. The mercapturic acid was obtained by acetylation of the S-(1-naphthyl)-cysteine. S-(1-naphthyl)glutathione was also confirmed by heating to 110°C in 6 N HCl for 24 hrs. The products were examined on tlc with solvent system butanol:acetic acid and water (11:4:5 v/v). Glycine, glutamic acid, and S-(1-naphthyl)cysteine were detected as ninhydrin-positive spots having R_f values of 0.23, 0.28, and 0.74, respectively.

S-(2,4-Dinitrophenyl)glutathione, S-(2,4-dinitrophenyl)cysteine and N-acetyl-S-(2,4-dinitrophenyl)cysteine were prepared from 2,4-dinitrofluorobenzene and their relative S-amino acid derivatives (Sokoloovsky et al., 1964 and Lamourex, 1975). The reaction mixtures were run in saturated sodium bicarbonate and 2 ml of ethanol solution. The crude products were obtained by adjusting reaction mixture to pH 2. The desired products were recrystallized several times from the crude products. Structural confirmation for the above compounds was performed by nuclear magnetic resonance and mass spectrometry. For the NMR spectra, samples were dissolved in D_2O containing 1% tetramethylsilane and measured with a Model-T-60 spectrometer (Varian Associates). Mass spectra were taken at 70 ev using a Finnegan Model 1015 C quadrupole mass spectrometer.

Radioassay and Chromatography - Radioactive samples were assayed for ^{14}C and ^{35}S by liquid scintillation counting using a Packard Tri-Carb scintillation spectrometer, Model 3314, equipped with automatic external standardization. The ether extractable and water soluble ^{14}C-metabolites were separated on tlc (Brinkman precoated silica gel 60-F-254, 250 mm) using the following solvent systems: (A) Ether:hexane 9:1; (B) Ether:hexane 3:7; (C) Chloroform:acetonitrile 2:1; (D) Hexane:benzene 1:1; (E) Benzene:acetic acid 95:5; (F) Butanol:acetic acid:water 11:4:5.

In Vitro Metabolism - Ten samples of fresh rat liver were homogenized with a Teflon pestle in a glass homogenizer containing 40 ml of 0.1 M adequate buffer pH. The homogenate was centrifuged at 15,000 g for 10 minutes. One-half of 15,000 g supernatant fraction was centrifuged at 105,000 for 1 hour to obtain the microsomal fraction and soluble supernatant. Three 4-ml samples of each fraction were used as the enzyme source. Control incubations were identical except that the homogenate was initially heated 30 minutes at 90°C. The incubation mixtures were then extracted with ether to remove unchanged substrates and unconjugated metabolites.

Animal Treatment - Six female Sprague-Dawley albino rats (average wt. 200 g) were used for each compound tested. The rats were given intraperitoneally a single dose of naphthalene-1-^{14}C or 1-naphthyl-1-^{14}C carbaryl at a concentration of 100 mg/kg and 25 mg/kg, respectively. The animals were housed in glass metabolism chambers where urine and feces were collected daily.

Analysis of Incubation Mixture and Urinary Radiocarbon - The incubation mixture was extracted with ether 3 times and the extracts concentrated to a volume which allowed direct application to silica gel thin-layer plates. Two-dimension solvent systems were used to resolve the radioactive components of the urine. Cochromatography with synthetic compounds and mass spectrometry were used to identify the metabolites.

The aqueous layer was lyophilized to dryness and the solid residue washed with methanol solution (contained 20% of water) repeatedly until no radioactivity was detected in the wash (10 ml x 3). The methanol wash was then concentrated for tlc analysis. Solvent system F was used to resolve the radioactive components of the aqueous layer. Extraction of each component detected by autoradiography from the plates was by scraping off the radioactive zone, sonicating with methanol solution, and then centrifuging to remove the gel.

Urine samples were extracted with ether and the aqueous layer divided into two fractions. The first fraction was handled as above while the second fraction was cooled to 10°C, adjusted to pH 2 with cold 2 N HCl and extracted with five equal volumes of ethyl acetate. The ethyl acetate was concentrated and chromatographed on tlc plates with solvent systems E and F.

Acid and Basic Hydrolysis of Water-soluble Metabolites - Portions of each water-soluble metabolite were incubated in 1 N HCl at 40°C for 24 hrs. The incubations were conducted in a sealed tube containing 4 ml of ether. Periodic shaking of the tubes extracted the hydrolyzed exocons (or non-polar products) into the ether layer to prevent further decomposition. The ether extracts were combined and analyzed by tlc. The aqueous solution was lyophilized to dryness and the solid residue washed with methanol solution. The methanol solution was then concentrated, and applied to tlc. Solvent system F was used to resolve the decomposition products.

Acid hydrolysis of glutathione conjugates was conducted in 50 µl of 6 N HCl, containing 20 µg of sample (glutathione conjugate or peptide amino acid). The mixture was contained in a 10-ml sealed tube and heated to 110°C for 24 hrs (Frear et al., 1973). Following hydrolysis, the reaction mixture was taken to dryness under reduced pressure. Basic hydrolysis was effected by heating the glutathione conjugates in 1 N NaOH solution (2 ml) at 40°C for 2 hr. In all hydrolysis, the exocons (or non-polar products) were isolated by ether extraction and subjected to tlc analysis.

Enzymatic Hydrolysis - Enzyme preparations were utilized to cleave possible conjugated water-soluble metabolites. The enzymes β-glucuronidase bacterial (powder), arylsulfatase (powder) and acetylase (from hog kidney) were purchased from Sigma Ltd. (St. Louis, Mo.). β-Glucuronidase hydrolysis of the conjugates (1.0 ml) was conducted in 0.1 M phosphate buffer pH 6.2 (2 ml) containing β-glucuronidase (10 units). The reaction mixture was incubated for 24 hr at 37°C.

Arylsulfatase hydrolysis was effected by incubation of 10 units for 24 hr at 37°C with the conjugates (1.0 ml in water) and 0.1 M sodium acetate-acetic buffer, pH 5.2 (2 ml). Acylase was handled in 0.1 M phosphate buffer pH 7.4 (2 ml) containing 5 units of acylase and conjugates. The reaction mixture was incubated for 16 hr at 37°C.

Amino Acid Determination - Ninhydrin reagent and the amino acid analyzer were used to detect amino acid or peptide conjugates. The ninhydrin reagent contained 300 mg of ninhydrin in 100 ml of 1-butanol. After the ninhydrin had dissolved, 3 ml of glacial acetic acid were added to the 1-butanol solution. After spraying the plates, color was developed by heating the plates for 10 to 20 min at 100°C. The Technicon® Sequential Multi-Sample Amino Acid Analyzer (TSM) was used to quantitate the amino acids, glutamic acid and glycine. The column operating conditions were following: flow rate 0.5 ml/min, temperature 60°C, resin bed 23.0-23.5 cm, resin type C-3.

Determination of Sulfur - Sulfur-containing compounds were located on tlc by spraying 0.1 M potassium dicromate and acetic acid (1:1 v/v), followed by 0.1 M silver nitrate (Knight and Young, 1958). The sulfur-containing compounds appeared as yellow spots on the plates.

Results and Discussion

Glutathione and Mercapturic Acid Conjugation of Naphthalene and Carbaryl in the Rat - Optimum substrate concentration and cofactors used in the metabolism of naphthalene-1-^{14}C and 1-naphthyl-1-^{14}C carbaryl *in vitro* were obtained from previous studies (Jerina et al., 1970; Bend et al., 1971). The amount of substrates used was 512 µg and 40 µg for naphthalene-1-^{14}C and 1-naphthyl-1-^{14}C carbaryl, respectively. Cofactors were the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH, 0.115 mg), magnesium chloride ($MgCl_2$, 6.41 mg), methylnicotinamide (6.34 mg), glucose-6-phosphate (G-6-P, 3.92 mg), glucose-6-phosphate dehydrogenase (G-6-PDH, 1 unit) and glutathione (1.0 mg).

The pH effect on conjugation was determined by the total amount of radioactivity transferred to the water layer after 30 min of incubation. Therefore, this assay included all types of conjugation and not just that attributed to glutathione. Different regions of optimum pH were observed for naphthalene-1-^{14}C (pH 8.0-8.5 for tris HCl buffer and pH 7.6-8.0 for phosphate buffer) and for 1-naphthyl-1-^{14}C carbaryl (pH 6.9-7.7 for phosphate buffer). This indicated that two different kinds of enzymes were involved in the conjugation of naphthalene and carbaryl.

The subcellular distribution of enzyme effective in the metabolism of naphthalene and carbaryl was investigated in an attempt to locate the fraction which processed the maximum enzymatic conjugative activity (Table 61). Two centrifugal fractions, one containing the 15,000 g supernatant and the other the 105,000 g precipitate plus 105,000 g supernatant, formed the highest amount of conjugation (above 60%) as compared to crude homogenate, microsomal and soluble fractions. About 15% conjugation was obtained in the incubation of naphthalene with microsomes as compared to 3.5% in the control incubation mixture; only 6.2% conjugation occurred with carbaryl as compared to 4.2% of the control incubation mixture. The 15% conjugation of naphthalene by

Table 61. CONJUGATION OF NAPHTHALENE-1-^{14}C AND CARBARYL BY DIFFERENT SUBCELLULAR FRACTIONS OF RAT LIVER HOMOGENATE[a].

	% Conjugation[b]	
Centrifugal fraction	Naphthalene	Carbaryl
Control	3.5	4.1
Crude homogenate	47.6	38.2
15,000 g Supernatant	72.2	64.7
105,000 g Precipitate (microsomal)	15.5	6.2
105,000 g Supernatant (soluble)	3.6	4.5
105,000 g Precipitate plus 105,000 g supernatant[c]	68.4	62.1

[a] Substrates: naphthalene 512 µg, carbaryl 40 µg, cofactors: NADPH 0.115 mg, magnesium chloride 6.41 mg, methylnicotinamide 6.34 mg, glucose-6-phosphate 3.92 mg. Glucose-6-phosphate dehydrogenase 1 unit, glutathione 1000 µg.

[b] Conjugation was taken as that quantity of radiocarbon converted to a water soluble form during incubation.

[c] 2 ml of 105,000 g precipitate and 2 ml of 105,000 g supernatant were used in the incubation mixture.

microsomes probably was catalyzed by GSH-S-transferase which was not completely removed from pellet.

The 15,000 g supernatant converted naphthalene and carbaryl to conjugation products to a greater extent than did the other fractions. This system was routinely used as a source for conjugation in additional studies. Attempts to obtain even greater conjugation by increasing the incubation time beyond 30 min were unsuccessful.

Formation of naphthalene conjugates by the 15,000 g supernatant increased from 34% to 74% upon addition of from 0-600 μg of glutathione, whereas formation of carbaryl and 1-naphthol conjugates was independent of additional glutathione. This indicated that glutathione conjugation occurred with naphthalene but not with carbaryl. It was also clear that glutathione reacted with a metabolite of naphthalene produced by the microsomes and not with the parent compound per se.

Metabolites Formed by Rat-Liver Enzymes - In order to determine the nature of metabolites from glutathione conjugation, the cross-labeled preparations of substrates were incubated separately with the 15,000 g supernatant for 10 minutes. The cross-labeled preparations of substrates contained five different sets of substrates in the experiment (Table 62). By using these combinations, it was possible to determine the presence or absence of the glutathione and naphthol moieties in the metabolites. The radiocarbon in each preparation was separated into three fractions and the water-soluble radiocarbon further separated by tlc. Eight different radioactive bands were observed. Incubation with naphthalene-1-^{14}C plus nonradioactive GSH and naphthalene plus GSH-^{35}S showed one metabolite (III) with the same R_f value and was, therefore, considered as a GSH conjugate. No metabolite with the same R_f value in the cross-labeled preparations of carbaryl and glutathione was observed; thus, no GSH metabolite of the carbamate was evidenced.

Table 62. NATURE OF RADIOCARBON FOLLOWING INCUBATION OF NAPHTHALENE-1-^{14}C AND 1-NAPHTHYL-1-^{14}C CARBARYL WITH 15,000 G SUPERNATANT OF A RAT LIVER HOMOGENATE.

Composition of incubation mixture	Distribution of radioactivity, % of that added to flask											
		Water solubles/R_f on tlc										
	Ether layer	I 0.00	II 0.23	III 0.44	IV 0.50	V 0.60	VI 0.72	VII 0.75	VIII 0.88	Total	Precip- itate	Total Recovered
A												
Naphthalene-1-^{14}C plus non-radioactive GSH	32.0	0	0	50.0	0	0	8.4	0	0	58.4	3.0	93.4
Naphthalene plus radio-active GSH-^{35}S	1.30	8.4	62.5	12.1	0	0	0	0	0	83.0	1.0	85.3
B												
Naphthyl-1-^{14}C carbaryl plus nonradioactive GSH	24.7	0	0	0	6.3	10.0	0	8.9	42.3	67.5	4.2	96.4
Carbaryl plus radioactive GSH-^{35}S	1.7	13.1	67.2	0	0	0	0	0	0	80.3	1.0	83.0
Carbonyl-^{14}C carbaryl plus nonradioactive GSH	26.5	0	0	0	7.6	8.6	0	0	43.8	60.0	4.5	91.0

Identification of the ether-soluble metabolites was attempted first, since information gained might help in the identification of conjugates. As shown in Table 62, 32% of the radiocarbon was extracted into ether from the incubation mixture using naphthalene-1-^{14}C and GSH. Four metabolites (two-dimensional solvent systems A and C) were detected in this extract (Table 63). The 1,2-dihydro-1,2-dihydroxy naphthalene accounted for 40% of the ether-soluble metabolites. The mass spectrum of this compound was complex but similar to a spectrum of 1-naphthol. The major parent ion, m/e 162, suggested incorporation of 2 oxygens and 2 hydrogens into the naphthalene ring. An ion of m/e 144, the molecular ion for 1-naphthol, and another ion at m/3 129 which showed the same abundance as m/e 128 suggested one more hydrogen relative to naphthalene. This indicated that the ion at m/e 129 was a hydro form of the naphthalene ring. 1-Naphthol (85%) and 2-naphthol (10%) were obtained after acid hydrolysis of 1,2-dihydro-1,2-dihydroxy naphthalene, indicating that a water molecule was removed from the original compound. The existence of 1,2-dihydro-1,2-dihydroxy naphthalene as a metabolite was consistent with previous findings (Jerina et al., 1970).

Ether-soluble metabolites obtained from incubation mixtures of 1-naphthyl 1-^{14}C carbaryl plus nonradioactive GSH were primarily 5,6-dihydro-5,6-dihydroxy 1-naphthyl N-methylcarbamate (28%) and 1-naphthyl N-hydroxymethylcarbamate (40%). Equal amounts of these two metabolites were also found by using carbonyl-^{14}C carbaryl plus nonradioactive GSH.

Water-soluble Metabolites - The identification of water-soluble metabolites I to VIII (Table 62) were attempted by enzymatic and acid hydrolysis (Table 64). Exocons released from the enzymatic and acid hydrolysis were identified by comparison of their tlc behavior and mass spectra with those of authentic standards. The characteristics of each water-soluble metabolite I to VIII are described individually as follows.

Table 63. RADIOACTIVE COMPONENTS OF THE ETHER FRACTION FROM *IN VITRO* METABOLISM OF NAPHTHALENE-1-^{14}C AND 1-NAPHTHYL-1-^{14}C CARBARYL

Metabolite	% of ^{14}C in sample		
	Naphthalene 1-^{14}C	Naphthyl-1-^{14}C carbaryl	Carbonyl-^{14}C carbaryl
Unknown origin material	3.1	2.0	1.8
5,6-Dihydro-5,6-dihydroxy 1-naphthyl N-methylcarbamate	-	28.3	30.1
1,2-Dihydro-1,2-dihydroxy naphthalene	40.6	-	-
1-Naphthyl N-hydroxy-methylcarbamate	-	40.5	41.4
Carbaryl	-	20.6	26.3
1-Naphthol	6.2	-	-
Naphthalene	43.7	-	-
Total recovery	93.6	91.4	99.6

[a] Ether fraction constituted 32.0%, 24.7%, 26.5% of the radiocarbon added for naphthalene-1-^{14}C, naphthyl-1-^{14}C carbaryl, carbonyl-^{14}C carbaryl, respectively.

- Indicates that no radiocarbon was detected.

Table 64. CONVERSION OF WATER SOLUBLE AND ETHER EXTRACTABLE MATERIALS BY ENZYME AND ACID TREATMENTS

Water soluble metabolites[a]	% Distribution of radioactivity after treatment with								
	Glucuronidase			Sulfatase			1 N HCl		
	Water	Ether	Total	Water	Ether	Total	Water	Ether	Total
I	97.6	1.1	98.7	90.5	1.0	91.5	84.2	3.6	87.8
II	97.5	1.4	98.9	93.4	1.3	94.7	88.8	3.5	92.3
III	94.8	1.4	96.2	95.9	1.1	97.0	81.2	7.1	88.3
IV	62.4	21.2	83.6	75.2	15.4	90.6	15.8	66.3	82.1
V	52.8	37.8	90.6	87.2	12.2	99.4	37.9	46.4	84.3
VI	12.4	78.9	91.3	74.7	18.8	93.5	41.3	54.4	95.7
VII	23.5	68.5	92.0	34.6	61.8	96.4	18.4	78.5	96.9
VIII	37.8	60.7	98.5	40.5	57.2	97.7	3.3	96.5	99.8

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[a] See Table 62 for position on tlc and source of metabolite.

I: This was tlc origin material. Obtained only from ^{35}S-labeled GSH plus naphthalene and carbaryl, and did not release ether extractable radiocarbon when subjected to enzymatic and acid hydrolysis.

II: Obtained only from ^{35}S-labeled, and not converted to ether-soluble materials by enzymatic and acid hydrolysis. It gave a positive response to ninhydrin reagent, and had a R_f value of 0.23 in solvent system F, which corresponds to that reported for oxidized glutathione (GSSG) by Shishido et al., 1972. Therefore, metabolite II was considered to be an oxidized form of glutathione.

III: Obtained from naphthalene-1-^{14}C plus nonradioactive GSH and could not be separated from the metabolite III (by tlc in solvent system F) obtained from naphthalene plus GSH-^{35}S when equal amounts of radioactivity from both sets of substrates were mixed together. This indicated that both metabolites obtained from different labels (^{14}C and ^{35}S) were identical.

The ^{14}C-metabolite III treated with glucuronidase and sulfatase converted no radioactivity to ether-soluble materials, and, thus, was not a glucuronic acid or a sulfate conjugate. A positive response to ninhydrin reagent was obtained as was a positive test to the $K_2Cr_2O_7$-$AgNO_3$ reagent, indicating that a free α-amino and sulfur were contained in the molecule. The metabolite chromatographed identically with synthetic S-(1-naphthyl)glutathione when treated with mild acid. This indicated that metabolite III was an acid labile compound, probably S-(2-hydroxy-1,2-dihydro 1-naphthyl)glutathione which was proposed by Jerina et al., 1970.

Tlc analysis of the 6N HCl hydrolysis mixture of metabolite III showed 5 radioactive compounds, plus 2 nonradioactive products, glutamic acid and glycine (Table 65). The major hydrolytic product, S-(1-naphthyl)-cysteine, was identified by comparison of its tlc behavior and mass spectra with that of authentic standards. S-(1-Naphthyl)cysteinyl

Table 65. PRODUCTS FORMED UPON TREATMENT OF 2-HYDROXY 1,2-DIHYDRO-S-(NAPHTHYL-1-^{14}C)GLUTATHIONE (III)[a] WITH 6 N HCL AT 110°C FOR 24 HR.

R_f of product[b]	% of ^{14}C applied to tlc	Tentative identification
1.00	4.4	1-Naphthol
0.74	52.2	S-(1-Naphthyl)cysteine
0.60	7.4	S-(1-Naphthyl)cysteinyl glutamic acid
0.51	13.1	S-(1-Naphthyl)cysteinyl glycine
0.46	7.3	S-(1-Naphthyl)glutathione
0.25[c]	-	Glutamic acid
0.16[c]	-	Glycine
Total ^{14}C	90.4	

[a] See Table 62 for position on tlc and source of metabolite.

[b] Reaction mixture was applied to 250 mm silica gel chromatoplates and developed in solvent system F.

[c] Products were located by ninhydrin spray.

glutamic acid. These two resultant products showed the incomplete cleavage of the peptide bond in the glutathione moiety. These characteristics were also found in the hydrolysis of the synthetic compound S-(1-naphthyl)glutathione. From the combined information, the chemical structure of metabolite III was considered to be S-(2-hydroxy-1,2-dihydro 1-naphthyl)glutathione.

IV: Obtained from 1-naphthyl-1-^{14}C carbaryl and carbonyl-^{14}C carbaryl and showed no separation of radioactivity by tlc in solvent system F when equal amounts of radioactivity from ring and carbonyl labeled were mixed together. Acid and enzyme hydrolysis gave 1-naphthyl N-hydroxymethylcarbamate. The radiocarbon which could not be hydrolyzed by acid was concentrated and reapplied to tlc. Solvent system F was used and unreacted original metabolite IV was obtained. The amount of hydrolysis by β-glucuronidase and sulfatase was similar (21% with β-glucuronidase and 15% with sulfatase). Therefore, it was concluded that metabolite IV consisted of glucuronide and sulfate esters of 1-naphthyl N-hydroxymethylcarbamate.

V: The metabolite was also obtained with carbonyl-^{14}C carbaryl (Table 62). This indicated that the carbamate ester was still intact as with metabolite IV. Acid and enzyme hydrolysis released the same exocon as did metabolite IV, i.e., 1-naphthyl N-hydroxymethylcarbamate. The radiocarbon which could not be hydrolyzed by acid was reapplied on tlc. The results showed that metabolite IV was observed in addition to unreacted original metabolite V. This evidence indicated that metabolite IV was derived from V by partial cleavage of the conjugate. While not confirmed experimentally, metabolite V may have been the N-hydroxymethyl analog conjugated both on the nitrogen and on the hydroxymethyl moiety. Thus, complete cleavage of the conjugate would give the same exocon as obtained with metabolite IV. But, cleavage of just the N-conjugate would yield metabolite IV directly.

VI: Metabolite VI derived from naphthalene was hydrolyzed by β-glucuronidase (79%) and sulfatase (18%) (Table 64). The exocon released from enzymatic hydrolysis was identified as 1,2-dihydro-1,2-dihydroxy naphthalene which was chromatographically and mass spectrally identical to 1,2-dihydro-1,2-dihydroxy naphthalene obtained as an ether-soluble metabolite. Acid hydrolysis gave two products, 1,2-dihydro-1,2-dihydroxy naphthalene and 1-naphthol. Their identities were confirmed by mass spectral analysis. Radiocarbon left in the aqueous layer after acid hydrolysis of metabolite VI showed two radioactive metabolites. They cochromatographed with the original metabolite VI and 1-naphthyl glucuronide. From the above evidence, metabolite VI was identified as 1,2-dihydro-2-hydroxy 1-naphthyl glucuronide.

VII: Metabolite VII was obtained from 1-naphthyl-1-^{14}C carbaryl plus nonradioactive GSH but was not obtained from carbonyl-^{14}C carbaryl. This indicated that the structure of metabolite VII did not contain the carbamate ester. Enzyme and acid treatment of metabolite VII yielded an exocon which chromatographed with 5,6-dihydro-5,6-dihydroxy naphthol. Its identity was confirmed by mass spectral analysis. The amounts of exocon released by treatment with glucuronidase and sulfatase were not significantly different (68% and 61% respectively). Therefore, the metabolite VII was considered to be 5,6-dihydro-5,6-dihydroxy 1-naphthyl glucuronide or sulfate.

VIII: Incubation of metabolite VIII with β-glucuronidase and sulfatase separately for 24 hr at 37°C cleaved 60% and 57% of the conjugate, respectively. Acid treatment yielded 96% ether-soluble materials which was in contrast to a control incubation containing all constituents except the acid where only 3% ether-soluble materials were found. The exocon released from the above hydrolysis was identified as 5,6-dihydro-5,6-dihydroxy 1-naphthyl N-methylcarbamate.

In summary, analysis of the water-soluble metabolites III and VI obtained from the metabolism of naphthalene-1-^{14}C _in vitro_ showed that glutathione conjugation was a major type of conjugation in the metabolism of naphthalene. On the contrary, glucuronic acid and sulfate conjugation were the major types of conjugation in the metabolism of carbaryl. Even though naphthalene and carbaryl have a similar phase I or primary metabolic pathway, formation of an epoxides and hydroxylations, their conjugation processes are quite different.

In Vivo Studies: Excretion - In the first 24 hr, the ^{14}C found in the urine was 62% of the administered carbaryl dose, while it was 24% for naphthalene. Total ^{14}C recovery from the rats dosed with naphthalene-1-^{14}C was 74% after 72 hr, with 60% in the urine and 14% in the feces. For 1-naphthyl-1-^{14}C carbaryl total ^{14}C recovery after 72 hr was 84%; 80% was in the urine and 4% in the feces.

Urinary ^{14}C Metabolites - Approximately 6% and 16% of the radiocarbon could be extracted by ether from the urine of rats dosed intraperitoneally with naphthalene-1-^{14}C and 1-naphthyl-1-^{14}C carbaryl respectively. In two dimension solvent system A and C, 3 metabolites from naphthalene-1-^{14}C and 8 metabolites from 1-naphthyl-1-^{14}C carbaryl were detected in ether extract (Table 66). 1,2-Dihydro-1,2-dihydroxy naphthelene (28%) and 1-naphthol (60%) were major ether-soluble metabolites in urine of rats dosed with naphthalene-1-^{14}C. 5,6-Dihydro-5,6-dihydroxy naphthol (27%), 4-hydroxy 1-naphthyl N-methylcarbamate (12%) and 5,6-dihydro-5,6-dihydroxy 1-naphthyl N-methylcarbamate (12%) were highest in the ether extract of urine of rats dosed with 1-naphthyl-1-^{14}C carbaryl. The occurrences of these ether-extractable metabolites in the urine of rats dosed with naphthalene-1-^{14}C and 1-naphthyl-1-^{14}C carbaryl were consistent with the previous studies (Boyland et al., 1958 and Dorough, 1970).

Four radioactive water-soluble metabolites (IX-XII) were isolated from the urine of rats given naphthalene-1-^{14}C intraperitoneally (Table 67). Metabolite XI was the major metabolite, accounting for 65% of total radiocarbon in the water-soluble fraction. Seven radioactive water-

Table 66. ETHER EXTRACTABLE RADIOACTIVE METABOLITES IN URINE EXCRETED DURING A 72-HR PERIOD AFTER RAT WERE TREATED WITH NAPHTHALENE-1-^{14}C AND 1-NAPHTHYL-1-^{14}C CARBARYL.

Metabolite	% of ^{14}C in ether[a]	
	Naphthalene	Carbaryl
Unknown origin material	2.1	10.5
5,6-Dihydro-5,6-dihydroxy 1-naphthyl N-methylcarbamate	-	11.9
1,2-Dihydro-1,2-dihydroxy naphthalene	28.3	-
3,4-Dihydro-3,4-dihydroxy naphthol	-	6.4
5,6-Dihydro-5,6-dihydroxy naphthol	-	27.0
5-Hydroxy 1-naphthyl N-methylcarbamate	-	7.0
4-Hydroxy 1-naphthyl N-methylcarbamate	-	12.0
Carbaryl	-	6.0
1-Naphthol	60.0	5.0
Total recovery	90.4	85.8

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[a] Percentage of ether extractable radiocarbon for naphthalene-1-^{14}C and 1-naphthyl-1- C carbaryl was 6.0 and 16.0 with respect to the administered dose.

- Indicates that no radiocarbon was detected.

Table 67. WATER-SOLUBLE RADIOACTIVE METABOLITES IN THE URINE EXCRETED DURING A 72-HR PERIOD AFTER RATS WERE TREATED WITH NAPHTHALENE-1-^{14}C.

R_f[a]	Designation	% of total radiocarbon in the water soluble fraction[b]
1.00	IX	5.2
0.87	X	8.0
0.81	XI	65.0
0.72	XII	16.8
Total Recovery		95.0

[a] Solvent system F was used

[b] Water soluble fraction contained 94.0% of radiocarbon in the total urine.

soluble metabolites were obtained from the urine or rat dosed with 1-naphthyl-1-^{14}C carbaryl (Table 68). The amount of each metabolite (XIII-XIX) ranged from 7% to 17%, but metabolite XVII was the major one. The identification of water-soluble metabolites IX to XIX was by comparison of the tlc behavior and mass spectra of exocons released by enzyme or acid hydrolysis (Table 69 and 70) with those of authentic standards.

IX: This metabolite(s) remained at the tlc origin and accounted for only 6% of the water-soluble fraction. No attempt was made to identify the product.

X: This metabolite was hydrolyzed by arylsulphatase; 77% of the conjugate was an exocon chromatographically and mass spectrally identical to 1,2-dihydro-1,2-dihydroxy naphthalene. Acidic hydrolysis of the metabolite X gave two exocons which cochromatographed with 1,2-dihydro-1,2-dihydroxy naphthalene and 1-naphthol in solvent system A. The radiocarbon which could not be hydrolyzed by acid was unreacted metabolite X. Based on the above evidences, metabolite X was apparently a sulfate ester of 1,2-dihydro-1,2-dihydroxy naphthalene.

XI: No ether-soluble exocon was released from this metabolite by β-glucuronidase and arylsulphatase, but ether-soluble radiocarbon (87%) was released by 3 N HCl (Table 69). Three compounds were obtained by using solvent system F and chromatography and mass spectroscopy showed that these three compounds corresponded to 1-naphthol, N-acetyl-S-(1-naphthyl)cysteine (R_f 0.90), and S-(1-naphthyl)cysteine (R_f 0.74) with percentages of 2%, 84% and 10%, respectively. The same compounds were obtained by basic hydrolysis, but with different percentages of each compound, 25%, 37% and 34%, respectively. The endocons which stayed in the water-soluble fraction after acid and base hydrolysis were located by spray agent. N-Acetylcysteine and cysteine were recognized as two endocons in the reaction mixture after metabolite XI was treated with acid and base. Acylase treatment of metabolite XI (ninhydrin negative) released an unknown compound with R_f o.72 which was ninhydrin positive.

Table 68. WATER-SOLUBLE RADIOACTIVE METABOLITES IN URINE EXCRETED DURING A 72-HR PERIOD AFTER RATS WERE TREATED WITH 1-NAPHTHYL-1-^{14}C CARBARYL.

R_f[a]	Designation	% of the radiocarbon in water-soluble fraction[b]
0.98	XIII	12.0
0.90	XIV	8.0
0.88	XV	14.0
0.84	XVI	7.0
0.81	XVII	17.4
0.75	XVIII	16.3
0.60	XIX	15.7
Total		90.4

[a] Solvent system F was used in this experiment.

[b] Water-soluble fraction contained 84% of radiocarbon in the total urine.

Table 69. CONVERSION OF WATER-SOLUBLE METABOLITE OF NAPHTHALENE-1-^{14}C FORMED IN VIVO BY RATS TO ETHER EXTRACTABLE MATERIALS BY ENZYME AND ACID TREATMENTS.

Water-soluble metabolite[a]	% Distribution of radioactivity after treatment with								
	Glucuronidase			Sulfatase			1 N HCl		
	Water	Ether	Total	Water	Ether	Total	Water	Water	Total
X	86.6	8.5	95.1	20.8	77.1	97.9	34.2	61.3	95.5
XI	95.2	4.3	99.5	94.5	4.2	98.7	10.0	87.0	97.0
XII	18.4	81.7	100.1	99.0	1.0	100.0	58.3	41.0	99.3

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[a] See Table 62 for position on tlc and source of metabolite.

Table 70. CONVERSION OF WATER-SOLUBLE METABOLITES OF 1-NAPHTHYL-1-^{14}C CARBARYL FORMED IN VIVO BY RATS TO ETHER EXTRACTABLE METABOLITES BY ENZYME AND ACID TREATMENTS.

Water-soluble metabolite[a]	% Distribution of radioactivity after treatment with								
	Glucuronidase			Sulfatase			1 N HCl		
	Water	Ether	Total	Water	Ether	Total	Water	Ether	Total
XIII	57.1	26.3	83.4	54.0	32.8	86.8	27.1	62.0	89.1
XIV	74.2	23.9	98.1	27.1	69.5	96.6	48.9	50.0	98.9
XV	37.5	55.3	92.8	72.0	21.3	93.3	27.3	62.4	89.7
XVI	65.4	29.8	95.2	49.7	50.0	99.7	24.6	78.4	93.0
XVII	48.2	47.0	95.2	75.0	20.2	95.2	24.0	65.1	89.1
XVIII	78.9	20.3	99.2	91.4	6.7	98.1	32.0	68.3	100.3
XIX	72.7	27.1	99.8	73.2	19.0	92.2	51.1	47.7	98.8

[a] See Table 62 for position on tlc and source of metabolites.

This unknown compound (R_f 0.72) was converted into S-(1-naphthyl)cysteine by acid hydrolysis. From the above evidence, this unknown (R_f 0.72) compound probably was S-(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine which was proposed by previous study (Boyland et al., 1958).

Further evidence for the identification of metabolite XI was obtained from the mass spectral data (Table 71). The mass spectra of metabolite XI analogs, such as N-acetyl-cysteine, cysteine, N-acetyl-S-(1-naphthyl)cysteine, and S-(1-naphthyl)cysteine were also obtained in order to assist in assigning the spectrum of metabolite XI. The relative abundance of M^+ and the prominent fragments, over m/e 40, of those analogs are listed in Table 71. The molecular ion of metabolite XI (m/e 264) was not seen. The major breakdown under electron impact was either loss of water to yield N-acetyl-S-(1-naphthyl)cysteine or loss of the N-acetyl-cysteine to yield naphthol (m/e 144, 12%). The resultant product, N-acetyl-S-(1-naphthyl)cysteine, was very unstable under electron impact. Two principle cleavages of N-acetyl-S-(1-naphthyl)cysteine under electron impact was proposed from the mass spectrum of N-acetyl-S-(1-naphthyl)cysteine and its analogs, N-acetylcysteine and S-(1-naphthyl)cysteine. The first cleavage was between the naphthalene and sulfur bond, to yield ions N-acetylcysteine m/e 163 (2%) and naphthyl ring m/e 128 (40%). The second cleavage was alpha to the naphthalene ring, to yield the 1-naphthalenethiol m/e 160 (30%) arising via a hydrogen transfer process. The prominent ion, at m/e 60, in the mass spectrum of metabolite XI was associated with fragmentation of N-acetylcysteine molecule.

From a previous study (Boyland et al., 1958), the structure of metabolite XI was not fully elucidated. It was generally identified by comparing the tlc behavior of its acid hydrolytic product with synthetic compound N-acetyl-S-(1-naphthyl)cysteine. Based on the above information, metabolite XI was identified as S-(1,2-dihydro-2-hydroxy 1-naphthyl)cysteine.

Table 71. THE MAJOR IONS IN MASS SPECTRA OF N-ACETYL-S-(1,2-DIHYDRO-2-HYDROXY-NAPHTHYL)CYSTEINE AND ITS ANALOGS.[a]

Cysteine[c]		N-Acetyl cysteine[c]		S-(1-Naphthyl)-cysteine[b]		N-Acetyl-S-(1-naphthyl) cysteine[b]		N-Acetyl-S-(1,2-dihydry-2-hydroxy-1-naphthyl) cysteine(XI)[a]		N-Acetyl-S-(1-naphthyl) cysteine[a]	
amμ	%	amμ	%	amμ	%	amμ	%	amμ	%	amμ	%
121	7	163	1.3	247	10	160	5.0	174	2	160	70
86	7	145	1.4	201	22	159	0.8	161	5	159	20
76	100	122	10	185	2	128	1.4	169	30	129	8
75	65	121	10	174	9	116	1.2	145	3	128	30
74	95	118	30	173	23	115	3.4	144	12	127	8
60	12	117	16	171	5	63	1.0	129	6	116	26
59	70	100	56	161	10	62	1.0	128	40	115	70
57	70	88	10	160	100	61	7.0	127	8	97	12
46	42	76	80	159	20	60	100	116	15	95	16
45	40	74	100	129	16	59	7	115	38	83	18
44	25	60	90	128	50	45	80	84	10	81	18
43	52	43	90	116	26	44	16	60	100	60	90
42	52	-	-	115	83	43	90	-	-	43	100

[a] Compounds obtained from the urine.

[b] Compounds obtained from laboratory synthesis.

[c] Compounds obtained from commercial source.

XII: Metabolite XII was identical to metabolite VI (1,2-dihydro-2-hydroxy 1-naphthyl glucuronide), obtained from metabolism of naphthalene *in vitro*.

The water-soluble metabolites (XIII to XIX) obtained from the urine of rats dosed with 1-naphthyl carbaryl were analyzed (Table 70) using the same techniques as those from the urine of rats dosed with naphthalene-1-^{14}C. The data obtained relative to metabolite XIII to XIX are described as follows:

XIII: 5,6-Dihydroxy-5,6-dihydroxy naphthol accounted for 50% of the radiocarbon in the ether fraction and 3,4-dihydro-3,4-dihydroxy naphthol accounted for 50%. The amounts of exocons released by β-glucuronidase and sulfatase (26% and 32% respectively) were similar. Therefore, metabolite XIII was tentatively identified as mixtures of glucuronide and sulfate conjugated forms of 5,6-dihydro-5,6-dihydroxy naphthol and 3,4-dihydro-3,4-dihydroxy naphthol.

XIV: Enzymatic and acid hydrolysis of metabolite XIV resulted in 23% ether-soluble materials with β-glucuronidase, 69% with sulfatase and 50% with acid. Two exocons in equal amounts were released from the hydrolyses and were identified as 4- and 5-hydroxy 1-naphthol N-methylcarbamate. Therefore, metabolite XIV was identified as glucuronide and sulfate conjugates of 4- and 5-hydroxy 1-naphthyl N-methylcarbamate.

XV: Metabolite XV was identical to metabolite VIII (5,6-dihydro-5,6-dihydroxy 1-naphthyl N-methylcarbamate glucuronide) obtained from the metabolism of 1-naphthyl-1-^{14}C carbaryl *in vitro*.

XVI: 1-Naphthyl sulfate was determined as the identity of metabolite XVI by identical tlc mobility of metabolite XVI and 1-naphthyl sulfate. Sulfatase hydrolysis (50% of conjugate) of standard 1-naphthyl sulfate and metabolite XVI substantiated that metabolite XVI was 1-naphthyl sulfate.

XVII: This metabolite was identified as the glucuronide of 1-naphthol by cochromatography with 1-naphthyl glucuronide. The exocon released from enzymatic and acid hydrolysis was 1-naphthol.

XVIII: Metabolite XVIII was identical to metabolite VII (5,6-dihydro-5,6-dihydroxy 1-naphthyl glucuronide and/or sulfate) obtained from the metabolism of carbaryl *in vitro*.

XIX: Metabolite XIX was identical to metabolite IV (1-naphthyl N-hydroxymethylcarbamate glucuronide/sulfate, obtained from the metabolism of carbaryl *in vitro*.

Results of acid hydrolysis of water-soluble metabolites XIII through XIX obtained from the urine of rats dosed with 1-naphthyl-1-^{14}C carbaryl substantiated those obtained by enzyme hydrolysis. Each water-soluble metabolite which remained in the aqueous layer after treatment with acid and enzyme was identified as the original water soluble metabolite. This indicated that mercapturic acid was not formed in the urine of rats dosed with carbaryl.

Previously, the mercapturic acid conjugate of bromobenzene was successfully isolated from rat urine after being adjusted to pH 2 (Gillham, 1968). The same procedure was used for detecting the mercapturic acid in the urine of rats treated with naphthalene-1-^{14}C and 1-naphthyl-1-^{14}C carbaryl. The results showed that N-acetyl-S-(1-naphthyl)cysteine, called 1-naphthyl mercapturic acid, was successfully isolated from the urine of rats treated with naphthalene-1-^{14}C (Table 72). The results also showed that the free dihyrodiol compounds, plus 1-naphthyl glucuronide and 1-naphthyl sulfate were extractable from the pH 2 urine of rats treated with 1-naphthyl-1-^{14}C carbaryl. This evidence indicated that no mercapturic acid derivatives of carbaryl were present in the urine of rats treated with the carbamate.

Table 72. RADIOACTIVE COMPONENTS IN THE ETHYL ACETATE OF URINE (ADJUSTED TO PH 2) EXCRETED DURING A 72-HR PERIOD AFTER RATS TREATED WITH NAPHTHALENE-1-^{14}C AND 1-NAPHTHYL-1-^{14}C CARBARYL

Naphthalene		Carbaryl	
Metabolite	% of total radioactivity in the urine	Metabolite	% of total radioactivity in the urine
N-Acetyl-S-(1-naphthyl)-cysteine	56.0	5,6-Dihydro-5,6-dihydroxy 1-naphthyl N-methylcarbamate	2.4
1-Naphthyl glucuronide	12.0	3,4-Dihydro-3,4-dihydroxy naphthol	6.5
		5,6-Dihydro-5,6-dihydroxy naphthol	8.7
		1-naphthyl sulfate	3.8
		1-naphthyl glucuronide	6.7
Sub Total	68.0	Sub Total	26.1
EtOAC Extract before adjust to pH 2	6.0		16.7
Remaining as water soluble	18.2		47.0
Total	92.2		89.8

In summary, the mercapturic acid conjugate was not found in the metabolism of carbaryl. Bend et al. (1971) claimed that when the water-soluble metabolites in the urine of rats dosed with carbaryl were treated with acid, the water-soluble metabolite left in the aqueous layer after acid hydrolysis was a thioether amino acid conjugate. The detailed procedure of Bend's was followed to generate the compound reported to be a thio amino acid conjugate. The compound gave an identical R_f value on tlc in solvent system F with metabolite XVIII. Hydrolysis of this "thioamino acid conjugate" to the extent of about 19% occurred with β-glucuronidase, about 17% occurred with sulfatase, and about 24% occurred with acid. 5,6-Dihydro-5,6-dihydroxy 1-naphthyl N-methylcarbamate (76%) and its hydrolysis product, 5,6,-dihydro-5,6,-dihydroxy naphthol (22%) were detected by chromatographic analysis of the extracts from enzyme and acid hydrolysis. These data proved that Bend's metabolite was not a thioether amino acid conjugate.

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VI SUMMARY

The current research on "Metabolism of Carbamate Insecticides" was conducted under a continuation grant (R-802005) sponsored by EPA for the period covering February 1, 1974 through January 31, 1977. A detailed progress report covering the prior project period was submitted in September 1972 and is identified as Report Number EPA-650/1-74-002. The present summary progress report consists of abstracts of published works and summaries of unpublished data resulting from research funded by EPA Grant Number R-802005 since 1973.

Isomerication and Beckmann Rearrangement Reactions in the Metabolism of Methomyl in Rats - Methomyl {S-methyl-N-[(methylcarbamoyl)-oxy]thioacetimidate}, also known as Lannate, may exist in two geometric configurations and the more stable *syn* [$^{14}C{=}N$] methomyl [$CH_3S(CH_3)C{=}NOC(O)NHCH_3$] was metabolized to respiratory $^{14}CO_2$ and $CH_3{}^{14}C{\equiv}N$ in a ratio of about 2 to 1. Studies with the *anti* isomer showed that it was metabolized predominantly to $CH_3{}^{14}C{\equiv}N$. These and other data are presented supporting the contention that *syn* methomyl is partially isomerized to the *anti* isomer in the animal prior to the hydrolysis of the ester linkage. After hydrolysis, the *syn* oxime [$CH_3S(CH_3){}^{14}C{=}NOH$] is further metabolized to $^{14}CO_2$ while the *anti* oxime is metabolized to $CH_3{}^{14}C{\equiv}N$. Proposed immediate precursors to the carbon dioxide and acetonitrile, formed by Beckmann rearrangement of the *syn* and *anti* oximes, are $CH_3S{}^{14}C(O)NHCH_3$ and [$CH_3{}^{14+}C{=}NSCH_3$]x^-, respectively.

Fate of Croneton (2-Ethylthiomethylphenyl N-Methylcarbamate) in Rats - The fate of Croneton, 2-ethylthiomethylphenyl N-methylcarbamate, was determined in rats following both single oral or dietary exposure to the ^{14}C-carbonyl- and ^{14}C-ring-labeled insecticide. Greater than 95% of the [^{14}C]-Croneton equivalents was excreted in the urine or as a combination of $^{14}CO_2$ (47%) and urinary products (41%) 72 hr after a single oral dose. The feces contained 2-7% of the dose. A similar excretion pattern was observed during a 7-day feeding period. The principal urinary metabolites were Croneton sulfoxide (23-28% of the dose), phenol sulfoxide

(20-23%), phenol sulfone (9-25%), and Croneton sulfone (3-11%) after a single oral dose and similar in the long term study. The carbamates were excreted primarily as free metabolites while the phenolic constituents were eliminated as acid labile conjugates. The 24-hr acute oral LD_{50} values of Croneton, Croneton sulfoxide, and Croneton sulfone to mice were 71, 59, and 282 mg/kg, respectively.

Fate of Croneton in Large Animals - A lactating Holstein cow and a male Yorkshire pig treated with a single oral dose, 0.5 mg/kg, of ring [^{14}C]-Croneton excreted 97.8% and 90.0% of the dose via the urine after 24 hr. Residues were not detected in swine tissues sampled 24 hr after treatment, and of the bovine tissues, only the kidney, liver and skin contained detectable radiocarbon (0.016, 0.017, and 0.05 ppm ^{14}C-Croneton equivalents, respectively). Milk collected 6 hr after treatment contained 128 ppb [^{14}C]-residues; 60% was as the free carbamate metabolites, Croneton sulfoxide and sulfone. White Leghorn hens given ring [^{14}C]-Croneton as a single oral dose (0.5 mg/kg) or as daily treatments for 7 days (0.5 mg/kg twice daily at 12-hr intervals) exhibited patterns of excretion and metabolism similar to that observed in the cow and pig. Birds sacrificed 4 hr after the last of the daily doses contained [^{14}C]-Croneton equivalents ranging from 0.019 ppm in the fat to 0.324 ppm in the kidney. By 24 hr, only the liver and kidney (0.444 and 0.022 ppm) contained residues in excess of 0.01 ppm, and these had declined to 0.01 ppm or less by 4 days. Residues in eggs were on the order of 0.03-0.04 ppm [^{14}C]-Croneton equivalents after 2 days of treatment and reached a maximum of 0.06-0.07 ppm after 7 days. They declined rapidly when treatment was terminated and were below detectable levels after 3 days. About 75% of the radiocarbon in the eggs was as free phenol sulfoxide and sulfone, 10% as free Croneton sulfoxide and Croneton sulfone, 5% as water soluble metabolites and 5% as unextracted residues. The remaining 5% was unknown metabolites in the organoextractable fraction.

Fate of Insecticides Administered Endotracheally to Rats - Administration of carbaryl, leptophos, parathion and chlordane as aerosols to rats via the trachea resulted in quantitative retention of the inhaled insecticides. None of the radioactivity was detected in the exhaled air except 2.5% of the ^{14}C-carbonyl carbaryl dose as carbon dioxide. Rapid ester hydrolysis of carbaryl, leptophos and parathion was evidenced by the different levels of radiocarbon in the blood following treatment with the same chemical, but with the radioactive carbon on the opposite sides of the ester linkage. The three compounds were eliminated in the urine in amounts greater than 90% of the inhaled doses by three days. With chlordane, excretion was primarily via the feces: 52% of the dose was eliminated after 6 days. It was concluded that the ultimate fate of these insecticides when inhaled was about the same as when ingested.

Insecticide Residues in Cigarette Smoke. Transfer and Fate in Rats - A quantitative smoking system was devised for mainstream smoke collection or for delivery to the lungs of rats via the trachea. Percentage transfer of ^{14}C-labeled insecticide equivalents to the mainstream smoke of cigarettes impregnated with five different ^{14}C-labeled insecticides (carbaryl, carbofuran, leptophos, DDT, and mirex) was approximately the same per milliliter of smoke, 0.2-0.3% of the ^{14}C-labeled insecticide contained in the burned tobacco, with puff volumes of 35 and 5 ml. Thirty-five milliliter puffs represent the average puff volume of man while a 5-ml puff was the volume administered to rats. Total recovery (ash, butt, sidestream smoke, and mainstream smoke) ranged from 88 to 102% of the ring-^{14}C-labeled insecticides added to that tobacco subsequently burned during the smoking process. Radioactive carbaryl, carbofuran, parathion, leptophos, and DDT were added to cigarettes and the mainstream smoke was directed to the lungs of rats via the trachea. Total radiocarbon transfer to the lungs ranged from 0 to 15% of that in the tobacco burned during a smoking process involving eight 5-ml puffs. Exhalation of ^{14}C-residues during this time was 24 to 30% of that inhaled with all insecticides except carbofuran, of which 42% of the residues was exhaled. After 5 hr, total exhalation of the consumed radiocarbon was 35% for parathion, 65% for carbofuran, and approximately 50% for the other products. The nature of the ^{14}C-residues inhaled, their urinary and fecal excretion, and their deposition in and dissipation from various organs and tissues are presented.

Chemical and Metabolic Characteristics of 1-Naphthyl β-D-Glucoside - 1-Naphthyl β-D-glucoside was stable under a variety of conditions encountered in metabolism studies. When 1-naphthyl-^{14}C glucoside was given orally to rats, 67% of the radiocarbon was eliminated in urine after 24 hr. Of this, 28% was the administered compound, 35% 1-naphthyl glucuronide, 15% 1-naphthyl sulfate, and 14% was 1-naphthol. Cleavage of the glucoside linkage was possibly the sole initial step in the metabolism of the compound. 1-Naphthol-^{14}C given as a single oral dose was eliminated from rats more rapidly, 90% of the dose in the 0-24-hr urine, than was its glucoside conjugate. 1-Naphthyl glucuronide constituted 81% of the radiocarbon while 17% was 1-naphthyl sulfate and only 1.6% was free 1-naphthol.

Glycoside Conjugative Activity in Different Insect and Vertebrate Species - The occurrence and comparative activity of glycoside conjugating enzymes were investigated in insect and vertebrate species. Although some quantitative differences were noted, all species readily conjugated the substrate, 1-naphthol. Glucosyltransferease activity was essentially the same in whole body homogenates and in selected tissues of the adult housefly, *Musca domestica L.*, and the American cockroach, *Periplaneta americana* (L.), and larvae of the alfalfa weevil, *Hyper postica* (Gyllenhal), tobacco hornworm, *Manduca sexta* (L.), and Indian meal moth, *Plodia interpunctella* (Hübner). In the rat, the comparable enzyme, glucuronyltransferase, was more active in the liver, kidney, and lungs than in the intestine, stomach, heart, fat, and brain. Glycoside conjugating enzymes were stable when sotred in the intact tissue or in various subcellular fractions at -20° and 0°C for 90 days. *In vitro* glycosylation of 1-naphthol by houseflies was not influenced by developmental state, age, sex or insecticide resistance.

Glucosylation by Housefly Microsomes and Effect of Monoamine Oxidase

Inhibitors - Glucosylation by housefly microsomes was studied using 1-^{14}C-1-naphthol as the substrate. With Tris-HCl buffer the optimum pH for the formation of naphthyl-β-D-glucoside was 8.5. Magnesium was the most effective divalent ion for activity when compared with cobalt, nickel and manganese. The apparent K_m for UDPG was 2×10^{-4} M and for 1-naphthol $5\text{-}7 \times 10^{-5}$ M. p-Nitrophenol was a competitive inhibitor for 1-naphthol glucosylation. The monoamine oxidase inhibitors, harmaline and tranylcypromine, non-competitively inhibited the conjugation of 1-naphthol.

Role of Glutathione Conjugation in Carbaryl Metabolism - *In vitro* and *in vivo* glutathione (GSH) conjugation in rats was investigated using 1-naphthyl-1-^{14}C carbaryl and naphthalene-1-^{14}C. Maximum *in vitro* conjugation (72%) occurred after incubation of naphthalene-1-^{14}C with the 15,000 g supernatant of a rat liver homogenate fortified with NADPH and GSH. The amount of conjugates formed was dependent on the amount of glutathione added. Microsomal enzymes were required for naphthalene to form GSH conjugates, demonstrating that oxidative metabolism preceded conjugate formation. Glutathione conjugates isolated by tlc included S-(1,2-dihydro-2-hydroxy 1-naphthyl)glutathione and 1,2-dihydro-2-hydroxy 1-naphthyl glucuronic acid. By comparing the nature of the glutathione conjugates with those obtained by the metabolism of carbaryl, it was concluded that glutathione conjugation did not occur with this carbamate insecticide. The same conclusion was drawn from studies using 1-naphthol. When naphthalene-1-^{14}C and 1-naphthyl-1-^{14}C carbaryl were injected into rats, 50 and 80% of the radioactivity was excreted in the urine within 72 hr, respectively. With naphthalene, 3 major metabolites were detected in the urine, with the mercapturic acid derivative accounting for 32% of the dose. In contrast, mercapturic acid metabolites were not evident in the urine of carbaryl-treated animals. Glucuronic acid and sulfate conjugates were the predominant types of metabolites found in the urine of rats dosed with carbaryl.

Bioavailability of Bound and Conjugated Carbamate Insecticide Residues -

Bound and conjugated forms of pesticides and/or their metabolites may occur as major terminal residues in plants destined for human consumption. The potential bioavailability of such residues in mammals was investigated by administering bound (radiocarbon remaining in the plant matrix after thorough solvent extraction) or conjugated (water soluble) plant metabolites of four ^{14}C-labeled methylcarbamate insecticides (carbaryl, carbofuran, aldicarb and Croneton) orally to female Sprague-Dawley rats. After two days 8.0 and 1.3% of the carbofuran and carbaryl bound-residue dose were eliminated in the urine, while 78.8 and 99.5% was voided in the feces. Only 1.4 and 0.4% of the respective bound insecticide treatment was found in the bile of biliary fistulated rats. When Croneton bound residue from sorghum was fed to rats, 16.4% of the dose was excreted in the urine and 90.8% in the feces after 48 hr, while only 2.5% was recovered in the bile of a bile duct-cannulated rat. Within 36 hr, the urinary excretion of the water-soluble metabolites of carbaryl, aldicarb, Croneton, and carbofuran was 87.7, 87.5, 81.2, and 64.8% of the dose. Radiocarbon equivalents in the feces were 9.7, 1.0, 3.7, and 8.3%, respectively. These data demonstrate that bound residues of the test compounds were very poorly absorbed from the gastrointestinal tract of rats, while the conjugated ones were readily absorbed.

Influence of Insecticide Exposure on the *In Vivo* and *In Vitro* Metabolic Activity of Rats - Insecticides were administered to rats as single doses, oral and IP, at approximately 1/10 their acute oral LD_{50} levels, or in the diet at concentrations where the daily intake was equivalent to the single doses. Carbofuran decreased the rate of urinary elimination of carbaryl-^{14}C from rats whereas Ruelene tended to increase the rates. However, the changes were minor, 12% or less, and the nature of the carbaryl metabolites was not altered. Carbaryl and carbofuran caused a reduction in liver protein of rats injected with the carbamates for five days, but had no affect when fed in the diet for 40 days. DDT increased the liver protein when injected but not when fed in the diet. When administered in combination, by injection or in the diet, DDT and carbaryl did not modify the protein content of the liver. Liver and kidney microsomes of rats fed DDT in the diet, alone or with carbaryl, resulted in increased oxidase activity. Neither of the compounds affected microsomal glucuronidation of 1-naphthol. In a four-week feeding study, the rate of weight gain was 15% less in animals fed carbaryl plus Ruelene or coumaphos plus Ruelene than in the control rats. No significant difference from the controls was observed with carbaryl or carbaryl plus carbofuran. Feeding these compounds did not affect urine pH; but the urea content was increased by all treatments and the glucose content increased by all except treatment with coumaphos plus Ruelene.

Influence of Dose on Carbamate Hydrolysis - An examination was undertaken of the relative roles played by the urinary and hydrolysis-dependent respiratory routes of elimination following various doses of ^{14}C-carbonyl Croneton. Hydrolysis inferred from $^{14}CO_2$ expiration from rats expressed in terms of the administered dose showed that relative hydrolysis was slowed and decreased with increasing dose. When rate of hydrolysis was examined graphically with time, biphasically exponential elimination of $^{14}CO_2$ was observed with a relatively fast α-phase lasting for about 12 hr followed by a generally slower β-phase. Half-times of the α-phase were nearly constant at low doses (about 4 hr at 0.2 or 2 mg/kg) but increase dramatically in good correlation with doses producing severe toxicity (14.5 hr at 100 gm/kg). Urinary excretion expressed as a percentage of the administered compound also declines at high doses. Administration of a moderate dose of atropine prior to Croneton did not alter hydrolysis, but increased urinary excretion.

Screening of Pesticides for Mutagenic Potential Using *Salmonella typhimurium* Mutants - The mutagenic activity of several pesticides and related analogues was examined with a set of four strains of *Salmonella typhimurium* (Ames Assay, TA1535 series; deep rough strains without excision repair). Nitrosocarbaryl, a derivative of the insecticide carbaryl, proved to be a potent base-pair substitution mutagen showing activity at 0.5 µg/plate, as well as demonstrating a relatively mild frameshift activity. Captan at 25 µg/plate showed both frameshift and base-pair substitution mutagenesis. The mutagenic properties of these two compounds decreased when exposed to rat liver homogenate. DDT, DDE, heptachlor, heptachlor epoxide, dieldrin, carbaryl, linuron, and diazinon were not mutagenic in this system. The *Salmonella typhimurium* strain TA1535 mutants had no significant effect upon the metabolism of carbaryl. Rapid conversion of carbaryl to water soluble metabolites indicated that the S-9 rat liver homogenate was a very efficient *in vitro* preparation. Detection of hydroxylated carbaryl intermediates common to known pathways suggested that the metabolism within the Ames test was representative of the *in vivo* mammalian metabolism. Addition of S-9 enzymes to the system resulted, therefore, in the mutagenic potential of the carbaryl metabolites being analyzed rather than just that of the parent compound as was the case in the absence of the liver preparation.